Characterization of putative sialidase genes in *Gardnerella vaginalis*

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By

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ABSTRACT

Gardnerella vaginalis is a hallmark organism in the dysbiosis bacterial vaginosis (BV) in reproductive age women although its role in this condition is not currently understood. Diversity within *G. vaginalis* in terms of virulence factors such as sialidase activity, may explain why it is also observed in asymptomatic women. This thesis aimed to identify genomic determinants of sialidase activity in *G. vaginalis* and better understand its role in BV.

G. vaginalis has demonstrated genotypic and phenotypic diversity in research over the years and has been divided into four subgroups (A-D) based on *cpn*60 universal target sequencing. Recent research has demonstrated that a previously identified sialidase gene (Gene 1) does not correlate with sialidase activity. Analysis of 39 available *G. vaginalis* genome sequences identified a second sialidase gene (Gene 2), and its presence correlated with sialidase activity in 112 *G. vaginalis* isolates. Based on examination of the predicted amino acid sequences of the two sialidases, we hypothesized that Gene 1 encodes an intracellular sialidase while Gene 2 encodes an extracellular sialidase found almost exclusively in subgroup B strains.

Gene 1 was shown to encode for a sialidase enzyme with a pH optimum of 4.5 to 5.0. No protein could be expressed from Gene 2 in *E. coli*. A homopolymer region was identified in Gene 2 that caused an early stop codon, and may be involved in slipped-strand mispairing. When sialidase activity was assayed in cultures of sialidase activity positive (Gene 2 positive) and negative (Gene 2 negative) isolates, activity was detected only in the cell pellet of Gene 2 positive isolates, suggesting that Protein 2 is a cell bound, extracellular sialidase.

The results of this thesis demonstrate that *G. vaginalis* has at least two sialidase genes, only one of which encodes the extracellular sialidase activity observed in some isolates. This may help explain why *G. vaginalis* is found in women with symptomatic BV, as well as women with no

signs of dysbiosis. The finding that extracellular sialidase activity is confined to subgroup B *G. vaginalis* suggests that these isolates may have an important role in the establishment and maintenance of vaginal dysbiosis.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ARDRA	Amplified ribosomal DNA restriction analysis
BLASTp	Basic local alignment search tool protein
BNR	Bacterial neuraminidase repeat
BV	Bacterial vaginosis
CBA	Columbia blood agar
CDC	Centers for Disease Control and Prevention
DNA	Dioxyribonucleic acid
DNTP	Dioxyribonucleotide triphosphate
EMBL-EBI	European Bioinformatics Institute
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
IMG	Integrated Microbial Genomes database
IPTG	isopropyl β-D-1-thiogalactopyranoside
NCBI	National Centre of Biotechnology Information
MUNANA hydrate	2'-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt
PCR	Polymerase chain reaction
RFU	Relative fluorescent unit
rRNA	Ribosomal ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STI	Sexually transmitted infection
UT	Universal target
UV	Ultraviolet

1 Introduction and Literature Review

1.1 Introduction

The vaginal microbiome plays a critical but vaguely understood role in women's health. The most common vaginal condition that affects women of reproductive age is bacterial vaginosis (BV), which is a shift from the healthy *Lactobacillus* dominated microbial community to an overgrowth of an anaerobic community (Verstraelen and Verhelst, 2009). BV is a dysbiosis that is strongly associated with negative health outcomes including troubling symptoms (Amsel et al., 1983), increased risk of sexually transmitted infections including HIV (Ugwumadu et al., 1997), and adverse pregnancy outcomes associated with preterm birth (Hillier et al., 1995). Causes of BV and abnormal vaginal microbiota are not fully understood and current diagnostics and treatment strategies are insufficient.

G. vaginalis has been considered a hallmark of BV and its detection has been relied on for the diagnosis of BV since its identification in 1953 (Gardner and Dukes, 1955; Leopold, 1953). *G. vaginalis* abundance is a large component of Nugent scoring, as it is one of three categories of cell morphotypes that contribute to the scoring system (Nugent et al., 1991). Despite the strong association with BV, *G. vaginalis* is detected in healthy women that report no symptoms typical of BV (Aroutcheva et al., 2001; Chaban et al., 2014; Ravel et al., 2011; Schellenberg et al., 2011; Tosun et al., 2007). In other words, these women may meet the microbiological definition of BV but not the clinical definition. Further complicating the definition of the role of *G. vaginalis* in the vaginal microbiome is its phenotypic diversity. Phenotypic diversity has been described in terms of putative virulence properties such as cytotoxicity (Gelber et al., 2008), biofilm formation

(Patterson et al., 2010) and sialidase activity (Lewis et al., 2013; Santiago et al., 2011).

Genotypic diversity in *G. vaginalis* has also been widely reported and described. Subgroups of *G. vaginalis* were first observed in a culture independent study characterizing vaginal microbiota based on PCR, cloning and sequencing of the chaperonin-60 (*cpn*60) universal target (UT) (Hill et al., 2005). Four clusters of *G. vaginalis* like *cpn*60 UT sequences were also observed in a subsequent, larger study of vaginal microbiota of African women that featured an early application of next generation sequencing of PCR amplicon libraries to the study of vaginal microbiota (Schellenberg et al., 2011). These observations were followed up with studies of cultured isolates that confirmed that the initial observations were not due to PCR or sequencing artefacts (Paramel Jayaprakash et al., 2012). Whole genome sequence comparisons of isolates representing the four *cpn*60-defined subgroups indicate that the subgroups meet the genomic criterion for definition as different species (Paramel Jayaprakash et al., 2012; Richter and Rossello-Mora, 2009). Ahmed et al. (2012) have independently described the same subgroupings, but this demonstration required the combined sequence data from 332 genes; a stark contrast to the 552 bp of *cpn*60 sequence required to resolve the same subgroups.

Application of next-generation sequencing technology to vaginal microbiome profiling has led to the identification of several "community state types" (CST) that account for the most common profiles observed in women studied to date (Ravel et al., 2011). These CST range from those dominated by *Lactobacillus* spp. to mixed communities associated with BV that are defined by a rich and diverse community of anaerobes, including *G. vaginalis* (Ravel et al., 2011; Walther-Antonio et al., 2014). We have identified additional CSTs using *cpn*60-based methods, which were not apparent in previous studies based on 16S rRNA gene sequences since they are defined by relative dominance of the *G. vaginalis* subgroups (Albert et al., 2015). These results, combined

with the apparently uneven distribution of *cpn*60-defined *G. vaginalis* subgroups among BV positive and negative African women, suggest that the resolution of these groups has clinical and diagnostic significance (Paramel Jayaprakash et al., 2012). Additional fuel for this hypothesis has been provided by our recent demonstration among a collection of 112 *G. vaginalis* isolates representing the four subgroups, sialidase activity is only detectable in subgroup B and occasionally in subgroup C (Schellenberg et al., 2016b).

While sialidase activity is widespread in bacteria due to its potential role in nutrition, it also serves as a virulence factor in many pathogenic bacteria (Corfield, 1992; Schellenberg et al., 2016b). In the vagina, sialidase enzymes can degrade sialoglycans including immunoglobulin A and vaginal mucins that normally provide an immunoprotective barrier (Wiggins et al., 2001). Sialidase activity is an important virulence factor associated with mucin degradation in BV and aerobic vaginitis (Donders et al., 2005) that subsequently contributes to adverse pregnancy outcomes (Mcgregor et al., 1994) and increased susceptibility to pathogens (Wiggins et al., 2001). Measurement of sialidase activity in vaginal fluid can be used as a diagnostic biomarker for BV (Myziuk et al., 2003) and preterm birth risk (Cauci et al., 2005). Although sialidase activity is commonly detected in *G. vaginalis*, the trait is not common to all isolates, and expression levels are highly variable among sialidase positive isolates (Lewis et al., 2013; Schellenberg et al., 2016b). Furthermore, the results of PCR assays targeting a putative sialidase gene annotated in whole genome sequence of *G. vaginalis* ATCC 14019 (protein accession ADP38272) have failed to support a connection between the presence of this gene and extracellular sialidase activity (Corfield, 1992; Pleckaityte et al., 2012; Santiago et al., 2011). An explicit link between the translated product of this putative gene sequence and actual enzymatic activity has yet to be made. Given the potential significance of sialidase activity in contributing to negative health outcomes

such as preterm birth, a more complete understanding of the genomic determinants of sialidase activity and their distribution among *G. vaginalis* subgroups is warranted.

The current diagnostic standard for BV is Nugent scoring, which is based on evaluation of a Gram stain of a vaginal swab sample. *G. vaginalis* "morphotypes" on the slide are counted as part of the calculation of the Nugent score (Nugent et al., 1991). If *G. vaginalis* is in fact at least four phenotypically distinct species, then treating it as a monolithic entity fails to acknowledge potentially significant diagnostic information that has implications for women's health. Using *cpn*60 sequence based methods offers a route to resolving subgroups within the context of the entire microbiome. To prove the value of this approach, elucidation of the ecological roles of the *G. vaginalis* subgroups and identification of biomarkers and virulence factors such as sialidase activity is essential.

1.2 Vaginal dysbiosis

1.2.1 Bacterial vaginosis: History and definitions

Bacterial vaginosis (BV) is a polymicrobial dysbiosis in the vaginal microbiome when the "healthy" community shifts from a *Lactobacillus* dominated one to an overgrowth of anaerobic organisms that includes *G. vaginalis, Bacteroides* spp., *Mobiluncus* spp. and *Mycoplasma hominis*. BV is characterized by troubling symptoms, such as thin, malodorous, grey/yellow discharge (Nugent et al., 1991). BV is associated with an increased risk of HIV and STI transmission (Marrs et al., 2012), and adverse pregnancy outcomes such as preterm birth and postpartum endometritis (Money, 2005). In early studies BV was known as "non-specific vaginitis", and it was not until after the second world war that there was a spike in studies and publications on the topic (Gardner and Dukes, 1955). Confusingly, the term "vaginosis" was used originally in 1964 to describe non-

microbiological cysts (Platt and Smout, 1964). The term was not used again until 1981, with bacterial association, to explain the overgrowth of *G. vaginalis* and other anaerobes, that was not characterized with typical inflammatory response which would be indicated by the suffix *–itis* (Holmes et al., 1981).

Despite an increasing understanding of pathogenesis and sequelae, the etiology of BV remains a mystery. Gardner and Dukes were "prepared to provide evidence that the vast majority of so-called 'non-specific' bacterial vaginitides constitute a specific infectious entity caused by a single etiological agent…. We have assigned the name *Haemophilus vaginalis*" (Gardner and Dukes, 1955). BV is now known to be a complex polymicrobial infection that not only involves *G. vaginalis* but many other anaerobic bacteria such as *Bacteriodes*, *Prevotella* and *Mobiluncus* species (Bradshaw et al., 2006). To complicate matters, *G. vaginalis* can also be found in seemingly healthy women that meet the microbiological definition of BV (high Nugent score) but do not report symptoms; this is known as asymptomatic BV (Dunkelberg, 1962; Schwebke, 2000).

There are some undefined atypical microbiota that are neither normal nor can they be considered BV. Aerobic vaginitis is characterized by an alkaline pH >6, yellow discharge, foul, rotten odour rather than the typical fishy smell associated with BV and the vagina may be red, inflamed or ulcerated (Donders et al., 2002). Aerobic vaginitis is accurately named with the suffix "*-itis*" due to the observation of vaginal ulceration and redness, which is distinct from BV where inflammation is not observed. Bacteria associated with aerobic vaginitis are mainly group B Streptococci, *Staphylcoccus aureus* and *Escherichia coli*. Donders et al. (2002) observed that these bacteria were present three to five times more frequently in cases of aerobic vaginitis than in women with *Lactobacillus* dominated vaginal microbiota. Sialidase activity has also been found to be a common factor between these two vaginal conditions (Marconi et al., 2013).

1.2.2 Diagnostic methods

Diagnostics for BV remain inadequate due the complex polymicrobial dynamic of the infection. Nugent et al. (1991) devised a standardized method that was reliable and economical using Gram stains of vaginal fluid and quantifying three different bacterial morphotypes present including *G. vaginalis* and *Bacteroides* species, *Lactobacillus*, and curved Gram-negative rods (Table 1.1). This method that Nugent proposed is a microbiological method that scores a Gram stain of vaginal fluid or mucus between 0-10 depending on the bacteria present. The resulting

Table 1.1 Nugent scoring rubric for microbiological diagnosis of BV

The examiner counts number of micro-organisms of each morphotype per field using a 100× objective on a Gram stain of a vaginal smear. The sum of the scores indicates the condition of health or dysbiosis. Scores 0-3 = normal, 4-6 = intermediate, 7-10 = BV.

Lactobacillus	SCORE	*Gardnerella, Bacteroides*	SCORE	Curved Gram Negative	SCORE
30 or >	0	0	0	0	0
5-30	1	<1	1	<1	1
1-4	2	1-4	2	1-4	1
<1	3	5-30	3	5-30	2
0	4	30 or >	4	30 or >	2

scale ranges from normal (0–3) through intermediate (score 4–6) to BV (score 7–10) (Table 1.1). In a 2009 review, it was stated that *G. vaginalis* is present in 50% of women with healthy vaginal microbiota (Livengood, 2009). Another microbiological method for evaluation of vaginal microbiota was described by Hay and Ison, who sought to simplify the Nugent score by analyzing Gram stained vaginal smears for the proportion of *Lactobacillus* rods and *Gardnerella* cocci (Ison and Hay, 2002). This resulted in a three-grade rubric: grade I = abundance of *Lactobacillus* (normal), grade II = equal amounts of *Lactobacillus* and *Gardnerella* (intermediate), grade III = abundance of *Gardnerella* (BV). When the two approaches were compared directly, grade III and grade I agreed with Nugent scores for BV and healthy microbiota, but there was discrepancy between smears that were scored 4-6 with Nugent as 47 % were grade III and 8 % were placed in grade I with the Hay and Ison's method. (Chawla et al., 2013). The two methods are very alike, however it is stated that the Hay and Ison's method may save time as it requires the operator to count cells and is required to identify fewer cell types when analyzing Gram stained slides (Chawla et al., 2013). Since the results of both methods reliably diagnosed BV Hay and Ison's method would be a good alternative in situations where time and expertise may be limited (i.e. mobile clinics) (Chawla et al., 2013).

Amsel's criteria are also commonly used for the clinical diagnosis of BV. To meet the criteria, women must exhibit three out of four signs: fishy odour on addition of potassium hydroxide to vaginal fluid (the "whiff" test), an alkali vaginal pH (pH > 4.5), thin grey/yellow discharge, and presence of clue cells (Amsel et al., 1983). Clue cells are vaginal epithelial cells that are coated in polymicrobial biofilm. Sha et al. (2005) conducted a comparison of Amsel's criteria, Nugent score and PCR detection of *G. vaginalis* for diagnosis of BV. Amsel's criteria, despite their simplicity, are relatively insensitive. Out of 203 BV positive samples (Nugent score

7-10), only 75 were BV positive by Amsel's criteria (Sha et al., 2005). The sensitivity and specificity of Amsel's criteria compared to Nugent scoring in this study was 37% and 99% respectively (Sha et al., 2005). Schwebke et al. (1996) reported sensitivity and specificity of 70% and 94% when comparing Amsel's and Nugent scoring. The Sha et al. (2005) study was conducted with an HIV positive cohort of women, which may contribute to the large difference of sensitivity of Amsel's criteria compared to the sensitivity seen in the Schwebke et al. (1996) study, as BV may present differently in women with HIV. The interpretation of symptoms and readings of different clinicians may also play a role. When we consider the results of these studies, Nugent scoring remains a convenient method and gold standard for BV diagnosis.

Other methods of diagnosing BV include PCR based screening and detection of genes and enzymes that are involved in virulence such as sialidase. Sialidase activity is not associated with a healthy *Lactobacillus* vaginal community, making it a target for diagnostics (Kampan et al., 2011). The BV Blue kit (Gryphus Diagnostics LLC, Knoxville, Tennessee) tests vaginal discharge for sialidase activity produced by BV associated anaerobic bacteria such as *G. vaginalis*, *Prevotella* and *Bacteroides* species (Kampan et al., 2011).

There has been development of molecular diagnostic methods that may be an improvement or replacement for Nugent scoring and Amsel's criteria. In a study conducted in 2009, Dumonceaux et al. (2009) applied multiplexed, bead-based flow cytometric detection and quantification of bacterial targets using the Bio-Rad Bioplex (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario) formerly known as the Luminex platform to diagnose BV from vaginal swab samples. This method allows for detection of many targets after a single PCR, making high-throughput analysis of multiple samples technically feasible (Dumonceaux et al., 2009). The use of this method coupled with highly discriminatory *cpn*60-specific probes allows for simultaneous

detection of *Lactobacillus* species and BV-associated bacteria (Dumonceaux et al., 2009). This study applied the Luminex method to characterize microbiota in a cohort of African sex workers. The results showed that individuals with normal vaginal microbiota as defined by microscopy had one or more species of *Lacobacillus* and undetectable levels of *G. vaginalis* or *Atopobium vaginae*, while African women with BV often had detectable levels of *G. vaginalis* and *A. vaginae*. This was also observed in vaginal samples from North American women (Dumonceaux et al., 2009). The sensitivity and specificity of the Luminex method was 90 % and 89 % and correlated well with Nugent scores (Dumonceaux et al., 2009).

Sha et al. (2005) used qPCR to determine the bacterial load of *G. vaginalis*, *Lactobacillus* and *Mycoplasma hominis* in vaginal swabs that were BV positive based on Nugent score as well as vaginal swabs that were BV negative based on Nugent score. This was done to determine the amount of each species that would differentiate the BV group from the non-BV group. BV vaginal swab samples were observed to have levels of *G. vaginalis* > 6.81 $\log_{10}$ bacterial counts/ml, *M. hominis* > 4.82 $\log_{10}$ bacterial counts/ml and *Lactobacillus* < 8.50 $\log_{10}$ bacterial counts/ml (Sha et al., 2005). High Nugent score (7-10) samples correlated with levels of BV-associated bacteria above the determined cut off point between BV and non-BV women. The sensitivity and specificity of this qPCR method and Nugent scoring were 78.4% and 95.6% respectively (Sha et al., 2005). This method offers a quantitative assessment of bacterial loads of BV associated organisms and detection of *M. hominis,* which lacks a cell wall and cannot be seen using Nugent scoring (Sha et al., 2005).

Other researchers have reported use of a multiplex PCR for detection of BV organisms including *G. vaginalis* and conclude it is an adequate diagnostic tool (Obata-Yasuoka et al., 2002). More recently Balashov et al. (2014) used PCR based methods for identification and quantification

of *G. vaginalis* and its clades/subgroups to determine association of clade with Nugent score and Amsel's criteria. This method allowed for identification of *G. vaginalis* bacterial loads and clade (subgroup) distribution in 60 clinical swab samples without culture based methods (Balashov et al., 2014). Balashov et al. (2014) illustrated that this molecular based method would be useful in BV diagnostics.

Nucleic acid based methods also allow for monitoring of treatment efficacy and predicting whether or not recurrence of BV is likely. Hilbert et al. (2016) used qPCR to monitor vaginal flora of 84 women diagnosed with BV by analyzing samples pre-treatment, 7-10 days post-treatment and 40-45 days post-treatment. Vaginal specimens were analyzed at these three time points using a panel of primers for BV associated bacteria with qPCR (Hilbert et al., 2016). The PCRs quantified DNA from BV associated bacteria such as *G. vaginalis*, *Atopobium vaginae, Leptotrichia*/*Sneathia*, *Megasphaera* phylotypes 1 and 2, and *Lactobacillus* species (*L. crispatus, L. gasseri, L. iners* and *L. jensenii*) (Hilbert et al., 2016). Out of 84 women, 77 were successfully treated with antimicrobials after 7-10 days, and 46 of these 77 women remained cured after 40-45 days while 31/77 developed recurrent BV. Hilbert et al. (2016) observed women with recurrent BV had higher concentrations of *Megasphaera* phylotype 2 at the initial diagnosis and greater vaginal pH, higher Nugent score, and greater concentration of *G. vaginalis* post-treatment. The use of multiplex panels in conjunction with Nugent scoring and Amsel's criteria provides details that allow identification of women who may be at risk for recurrence of BV. Nucleic acid based methods allow species-specific detection of multiple taxa at once, which provides more detailed information than phenotypic methods alone for understanding the diversity and richness of the vaginal microbiota during BV.

1.2.3 Asymptomatic BV

Gardnerella vaginalis is strongly associated with diagnosis of BV, however it is also commonly found in women who do not report symptoms of vaginal dysbiosis (Shipitsyna et al., 2013; Totten et al., 1982). A study conducted in the USA that sampled over 3700 women over a 3-year period reported that 30 % of women had BV based on Nugent scores, but only 15 % of these women reported having symptoms (Koumans et al., 2007). During the initial characterization studies of *G. vaginalis*, Gardner and Dukes inferred that the microorganism might be present in women but fails to cause clinical symptoms. They did not consider, however, that it may be part of the "normal" vaginal microbiota, and instead characterized *H. vaginalis* as a surface parasite without invasive tendencies (Dunkelberg, 1962). Ray and Maughan (1956) examined 447 patients, of which 68 were clue cell positive, which at the time indicated BV or vaginitis; 19 of them were asymptomatic. They concluded that *H. vaginalis* is an organism of low virulence when unassociated with yeasts or *Trichomonas* (Maughan, 1956). Half of all women diagnosed with BV based on Nugent score typically do not report symptoms but do meet Amsel's clinical criteria of BV (Schwebke, 2000). The lack of symptoms may be due to poor recognition by the affected women (Amsel et al., 1983; Schwebke, 2000). Women who are diagnosed with BV based on Nugent score but do not have signs to meet Amsel's criteria and report no symptoms face the predicament of whether or not to treat the condition. There continues to be debate as to whether asymptomatic women should be treated for BV.

More recent studies have examined the effects of treatment of asymptomatic BV to collect data to explain the natural history of the condition (Dunkelberg, 1962; Schwebke, 2000; Schwebke and Desmond, 2007a, b). More data is needed to determine if asymptomatic BV resolves itself, remains stable, or evolves into symptomatic BV (Schwebke and Desmond, 2007a). Kero et al.

(2017) conducted a study looking at the association of asymptomatic BV with genital human papilloma virus (HPV) persistence and prevalence. There were 329 asymptomatic women enrolled in the study, and BV was detected in 12 % of the cohort, while 57 % had mixed microbiota, inconsistent with either health or BV (Kero et al., 2017). In HPV-positive women, BV but not yeast infection was a significant covariate of HPV persistence ($p = 0.024$; OR 2.15, 95% CI 1.13–4.08). Since BV was indicative of HPV persistence, the authors proposed that treatment of asymptomatic BV would be beneficial for those women (Kero et al., 2017).

Schwebke and Desmond (2007b) examined the effects of treatment on acquisition of STIs in a cohort of women diagnosed with BV based on Nugent score but who did not report symptoms of vaginal odour or discharge. A randomized treatment trial was performed with 107 women: 54 were assigned to observation and 53 were assigned to metrodinazole treatment and observed at regular intervals for the development of an STI. The median time to development in observational women was 94 days while in the treatment group it was 138 days and this difference was statistically significant (Schwebke and Desmond, 2007b). In another study conducted in the late 1980s, BV was often seen as a co-infection with cervical and vaginal STIs (Thomason et al., 1988). Women with trichomoniasis are highly likely to be co-infected with BV (Thomason et al., 1988; Wolner-Hanssen et al., 1989). Acquisition of trichomoniasis, *Neisseria gonorrhoeae*, and *Chlamydia trachomatis* has been associated with abnormal vaginal microbiota (Schwebke and Desmond, 2007b).

Treatment and prophylaxis for asymptomatic BV is associated with lowered acquisition rates of STIs (Schwebke and Desmond, 2007b), and there is evidence that treatment of asymptomatic BV is beneficial to women in reducing risk of STI/HIV and recurrent BV (Sobel et al., 2006). Despite many studies showing that abnormal microbiota is a risk factor for STI/HIV

transmission, and that treatment of asymptomatic BV reduces risk of STI acquisition, treatment of women with asymptomatic BV is generally not recommended by the Centers for Disease Control (CDC). The CDC states that evidence of treating asymptomatic BV in pregnant women is inconsistent, as some studies report there being a benefit, some report no benefit and one even reports harm and thus cannot recommend treatment of pregnant women with asymptomatic BV (CDC, 2015). Similarly the Society of Obstetricians and Gynecologists of Canada do not recommend treatment for asymptomatic women or women with identified risk factors for preterm birth (CDC, 2015). The Public Health Agency of Canada states in the Canadian Guidelines on Sexually Transmitted Infections that treatment is unnecessary for asymptomatic women except in cases where there is high risk for preterm birth, or prior to insertion of an intrauterine device, or before gynecologic surgery, therapeutic abortion or upper tract instrumentation (Public Health Agency of Canada, 2010).

Treatment strategies listed in the 2015 guidelines published by the CDC that are commonly prescribed to women diagnosed with BV include oral metronidazole (500mg twice a day for 7 days), metronidazole 0.75% gel (intravaginal (5g) once a day for 5 days), or clindamycin 2% (intravaginal (5g) once a day for 7 days (CDC, 2015). The Public Health Agency of Canada recommended treatments for symptomatic BV are the same as those listed by the CDC. Alternative treatments include tinidazole (2g orally once daily for 2 days), tinidazole (1g orally once daily for 5 days), or clindamycin (300mg orally twice daily for 7 days) or clindamycin ovules (100mg once at bedtime for 3 days) (CDC, 2015). The recommended treatment for recurrent BV is metronidazole (500mg orally once per day for 10 -14 days), or metronidazole 0.75% gel (5g intravaginal once per day for 10 days) (Public Health Agency of Canada, 2010). Other therapies that are occasionally implemented in cases of recurrent BV include intravaginal boric acid (600mg

once per day for 21 days), in combination with metronidazole 0.75% gel twice weekly for 4-5 months. There is limited data on the effectiveness of this method (CDC, 2015).

1.2.4 Association of *Gardnerella vaginalis* with BV

Gardnerella vaginalis has been associated with what was described as non-specific vaginitis, now known as BV, since its discovery in 1953. *G. vaginalis* has continued to be associated with BV as seen in diagnostics and is thought to have an important role in the development of BV. The main diagnostic for BV used today is Nugent scoring of Gram stained vaginal swabs. This method allows visualization of bacterial and epithelial cells from the vagina, looking for sparse *Lactobacillus* rods as would be observed on a healthy Gram stain, or epithelial cells with high numbers of bacterial cells adhered to them via biofilm (clue cells) consistent with the presence of BV (Nugent et al., 1991). The association of *G. vaginalis* with BV is illustrated in epidemiological or clinical studies as well as DNA sequencing based studies.

G. vaginalis has been associated with diagnosis of BV for many years, using selective culture and Gram stain methods. Gram stain of vaginal fluid has been used as laboratory confirmation of BV since 1965, when Dunkelberg (1965) examined 300 vaginal smears and found that all 132 women with BV had consistent Gram stain results based on the presence of clue cells. Spiegel et al. (1983) conducted a study to compare Gram stain analysis of vaginal microbiota with clinical observations. Spiegel et al. (1983) analyzed Gram stains of 60 women with BV and found perfect agreement with clinical signs and detection of *G. vaginalis* on Gram stain of vaginal fluid. Based on these findings Spiegel et al. (1983) suggests Gram stain could be a useful tool in diagnostics of BV to complement clinical examinations or be used on its own. In a clinical study in the United States, 117 women were recruited, 32/117 were BV positive. *G. vaginalis* was

isolated from 28/32 BV positive samples by streaking specimens onto selective media (Aroutcheva et al., 2001). While selective culture of *G. vaginalis* from vaginal samples has excellent sensitivity for diagnosis of BV, the predictive value is < 50% making culture an inadequate diagnostic (Hillier, 1993).

Associations of *G. vaginalis* with BV have been further supported by culture-independent studies. For example, in a study that utilized *cpn*60-based profiling seven CSTs were observed in a cohort of 310 healthy, non-pregnant reproductive aged women (Albert et al., 2015). Two of these CSTs: IVC and IVD, were defined by a high proportion of *G. vaginalis* and the detection of these CST correlated with positive BV diagnosis based on Nugent scores (Albert et al., 2015). Overall, most women with these CSTs were BV positive, in that 45% of women with IVC and 67% of women with IVD were BV positive (Albert et al., 2015). Similarly, the vaginal microbiomes of BV positive East African women, were characterized by high diversity and high abundances of *G. vaginalis*, Bacteroidetes and Clostridiales (Schellenberg et al., 2011).

1.3 *Gardnerella vaginalis*

1.3.1 History and classification

Gardnerella vaginalis is a pleomorphic, non-motile, Gram-variable coccobacillus in the phylum Actinobacteria, and the family Bifidobacteriacea. Previously named *Haemophilus vaginalis* in 1953 (Gardner and Dukes, 1955; Leopold, 1953), it has undergone scrupulous analysis to define it taxonomically throughout the decades. In the 1970s it was proposed that *H. vaginalis* be renamed *Corynebacterium vaginale* (Dunkelberg et al., 1970; Piot et al., 1980) however the cell wall of *H. vaginalis* is distinct from true corynebacteria (Criswell et al., 1971; Piot et al., 1980). *Lactobacillus* had also been proposed as a possible niche for *H. vaginalis*, but it was thought

unlikely to belong to this genus or to *Eubacterium,* or *Propionibacterium* as acetic acid is the fermentation end-product as opposed to lactic, butyric, or propionic acids (Piot et al., 1980). Clearly *H. vaginalis* formed a distinct taxospecies that had little similarity with established Gram-positive or Gram-negative genera. In the late 1970s it was proposed that this organism be classified in its own genus, *Gardnerella,* named after H.L. Gardner (Greenwood and Pickett, 1979; Piot et al., 1980; Schellenberg et al., 2016a). Classifying the organism into the genus *Gardnerella* still did not address the observation and isolation of the organism from women with asymptomatic vaginitis (Dunkelberg, 1962). This is indicative that the criteria used to classify this organism did not provide the specific resolution between pathogenic and non-pathogenic strains of *G. vaginalis*, which is also observed in other organisms such as *Escherichia coli* and *Staphlococcus aureus* where there are pathogenic and non-pathogenic strains. In studies following, additional demonstrations of genotypic and phenotypic diversity within *G. vaginalis* have occurred and most recently, the analysis of *G. vaginalis* using whole genome sequencing or sequencing of the *cpn*60 UT show support for the division of *G. vaginalis* into four species (Ahmed et al., 2012; Paramel Jayaprakash et al., 2012; Schellenberg et al., 2016b).

1.3.2 Phenotypic diversity

There is a plethora of diversity within *Gardnerella vaginalis* that has begun to be recognized and appreciated, leading scientists to try to illustrate it using phenotypic and genotypic differences. Variation has been observed in the absence or presence of virulence factors that aid in the establishment of *G. vaginalis* in the vaginal environment; among these factors are sialidase, prolidase, lipase, haemolysin, mucinase and biofilm formation. Piot et al. (1984) defined eight different biotypes of *G. vaginalis* through testing for hippurate hydrolysis, lipase and β-galactosidase activity. These biotypes did not illuminate the difference in virulence between them

possibly due to markers selected, thus no single biotype could be associated with BV, and the appearance of multiple biotypes in 14% of women also occluded this analysis. An acknowledged limitation of the study by Piot et al. (1984) was that the analysis of only four isolates from each woman likely led to an underestimation of the proportion of women hosting multiple biotypes. Women with BV were found to be very likely to have a male sexual partner with the same biotype of *G. vaginalis* in his urethral microbiome suggesting that *G. vaginalis* is sexually transmitted (Piot et al., 1984). A mere two years later Benito et al. (1986) modified this biotyping method and identified 17 biotypes from 197 *G. vaginalis* strains isolated from asymptomatic and symptomatic women with BV. These biotypes were based on Piot's methods in addition to assessment of fermentation of xylose, arabinose, and galactose (Benito et al., 1986).

Reports of correspondence of *G. vaginalis* biotype with BV status have been mixed. Piot et al. (1984) did not observe any association of biotype isolated from BV or non-BV women, while others have reported that lipase-positive biotypes 1, 2, 3 and 4 predominate in women with BV (Briselden and Hillier, 1990). While using their modified version of the Piot et al. (1984) biotyping scheme, Benito et al. (1986) observed that biotypes 2, 4, 5 and 7 were predominant in women with BV. Some later studies also have shown some of the biotypes of *G. vaginalis* from both the Piot and Benito biotyping schemes are associated with BV (Aroutcheva et al., 2001). Piot biotypes 1, 4 and 5 are the most often isolated regardless of BV status (Tosun et al., 2007) and biotype 5 has been mainly associated with healthy vaginal microbiomes (Aroutcheva et al., 2001). Piot biotypes 7 and 8 have been observed most frequently in BV patients (Aroutcheva et al., 2001). Nath et al. (1992) found that 12/76 samples from women with BV contained more than one biotype (eleven samples had two biotypes, and one sample had three biotypes detected), indicating that some BV cases involve a mixture of *G. vaginalis* biotypes. Overall, biotyping methods have not shown

consistent associations between biotype and BV status, suggesting that the traits assessed are not significant in the pathogenesis of BV. Another critical limitation of these approaches is that they require culture of *G. vaginalis* isolates, which increases the time it takes to get results, limiting diagnostic utility.

In addition to using broad phenotypic categories like biotypes, attempts have also been made to associate more specifically defined *G. vaginalis* phenotypes with BV. Biofilm formation by *G. vaginalis* has received particular attention due to the importance of biofilm in the definition of BV. Although it is clear that biofilm formation by *G. vaginalis* is a critical aspect of BV, using biofilm formation as a characteristic for resolution of pathogenic and non-pathogenic *G. vaginalis* is problematic due to the variability of biofilm formation observed depending on culture media (Paramel Jayaprakash et al., 2012). In a small study of two *G. vaginalis* isolates, one isolated from a BV case and one from a healthy individual, a BAP family gene that is predicted to encode a protein associated with biofilm formation was identified in both isolates and may influence amount of biofilm (Harwich et al., 2010). A comparison of biofilm formation was conducted to determine if formation differed between the BV and the non-BV isolates. Upon observation of biofilm formation, the BV isolate had significantly more biofilm production than the non-BV isolate, indicating there may be genotypic difference that allows one to flourish under BV conditions (Harwich et al., 2010). The media in which these isolates were grown and tested for biofilm formation was not stated, so the influence of media cannot be discerned. Biofilm behaviour of cultured isolates in the laboratory may not be a good indicator of biofilm forming potential in the complex environment of the vaginal microbiome, where *G. vaginalis* has been observed in multi-species biofilms (Hardy et al., 2017a).

1.3.3 Genotypic diversity

Phenotypic methods can be highly variable and are not fully reliable in epidemiological studies of bacteria (Ingianni et al., 1997). It is important to have reliable phenotypic tests if they are going to be relied upon to consistently identify bacterial strains since variation in phenotypic traits used for characterization can lead to misidentification of strains. This limitation has led to exploration of genotypic methods to identify and differentiate bacteria more consistently.

Early attempts to classify *G. vaginalis* strains based on genotype relied on methods employing restriction enzyme digestion, such as restriction enzyme analysis (REA) and restriction fragment length polymorphism (RFLP). The principle of REA is to differentiate DNA based on the restriction enzyme sites. Changes in DNA sequence associated with speciation can lead to changes in restriction enzyme cleavage sites, resulting in a different pattern between closely related genomes when they are digested with particular restriction enzymes. RFLP uses restriction sites and a DNA probe to identify differences in restriction fragment lengths produced by digestion of genomic DNA from different bacteria. In a study by Nath et al. (1992) that implemented REA and RFLP for characterizing genotypic differences in BV associated *G. vaginalis* isolates no correlation between biotype and DNA fingerprint of *G. vaginalis* isolates was observed and no specific genotype or biotype of *G. vaginalis* was associated with BV.

Amplified ribosomal DNA restriction analysis (ARDRA) is a method of genotyping based on restriction enzyme digestion of PCR amplified 16S rRNA gene sequences (Vaneechoutte et al., 1993), which has been applied to characterization of *G. vaginalis* (Ingianni et al., 1997). Ingianni et al. (1997) conducted ARDRA with different restriction enzymes. When the 16S rRNA amplicon was digested with *Taq*I three distinct band patterns were detected and designated genotypes A, B

and C. Analysis of the 16S rRNA gene digested with *Hpa*I produced four genotype patterns, genotypes 1, 2, 3, and 4. Results from both enzymes were overlapping, except for isolates in genotype 4, which were found in genotype A, B and C (Ingianni et al., 1997). No correlation of genotype and geographic origin of the isolates was observed, and no genotype could be linked with BV or asymptomatic BV based on these observations (Ingianni et al., 1997). This was consistent with previous findings that no *G. vaginalis* strain involved in BV could be characterized with a particular genomic subtype (Nath et al., 1992). The Ingianni et al. (1997) study did not attempt to link these observed genotypes with previously described biotypes of these specific isolates. Other studies have detected only genotypes 1 and 2 using ARDRA, while genotype 3 was not detected. A potential explanation for genotype 3 being undetected is low sample size, however genotype 3 appears as a combination of genotypes 1 and 2 and the pattern may be the result of analysis of a mixed culture of genotype 1 and 2 strains (Pleckaityte et al., 2012; Schellenberg et al., 2016b). Similar to studies attempting to show a correspondence of specific biotypes and BV symptoms (Briselden and Hillier, 1990; Tosun et al., 2007), the association of ARDRA genotypes with biotype or certain virulence factors has also been variable (Paramel Jayaprakash et al., 2012; Pleckaityte et al., 2012; Santiago et al., 2011; Schellenberg et al., 2016b).

Accessibility and decreasing costs of DNA sequencing technology has opened opportunities to apply these approaches to the challenge of describing diversity in *G. vaginalis*. Ahmed et al. (2012) combined data from whole genome sequencing of 12 clinical *G. vaginalis* isolates and five previously sequenced *G. vaginalis* isolates to examine genomic diversity within the species. Isolates included in the study varied in clinical site of isolation (vagina and endometrium), co-morbid conditions, patient symptoms, biotype and Nugent score (Ahmed et al., 2012). Based on analysis of concatenated sequences of 473 protein-encoding genes found in all 17 genomes, four subgroups were observed (Ahmed et al., 2012).

A much earlier study using *cpn*60 UT sequencing had also identified four clusters of *G. vaginalis* with a range of 89 to 100 % identity to the type strain ATCC 14018 (Hill et al., 2005). Hummelen et al. (2010) also observed *G. vaginalis* as four groups, illustrated by the difference of a single nucleotide in the short 16S rRNA V6 region, but this study did not include sequence data from any cultured isolates to confirm the observation. At the same time the Ahmed et al. (2012) paper was published, a paper following up on the Hill et al. (2005) observations demonstrated the resolution of four *G. vaginalis* subgroups using *cpn*60 UT sequencing of cultured isolates and providing an in-depth investigation into the genotypic separation of *G. vaginalis*. Paramel Jayaprakash et al. (2012) looked at *G. vaginalis* ATCC 14018 and 49145 as well as eight clinical isolates from Kenyan and Canadian women. These isolates were tested for Piot biotype, ARDRA genotype, biofilm formation, and phylogenetic analysis of *cpn*60 UT sequences (Paramel Jayaprakash et al., 2012). There was discrete separation of *G. vaginalis* into four subgroups, supported by bimodal distribution of pair wise distances (inter- and intra-subgroup) between strains (Figure 1.1) (Paramel Jayaprakash et al., 2012). The four resolved subgroups were

designated A, B, C and D (Paramel Jayaprakash et al., 2012). Subsequent analysis of whole genome sequences and *cpn*60 universal target sequences has been used to confirm that the four "clades" identified by Ahmed et al. (2012) are the same groupings as the four *cpn*60-based subgroups (with subgroup A corresponding to clade 4, subgroup B to clade 2, subgroup C to clade 1 and subgroup D to clade 3) and furthermore, that the subgroups meet the genomic requirements to be considered separate species (Schellenberg et al., 2016b).

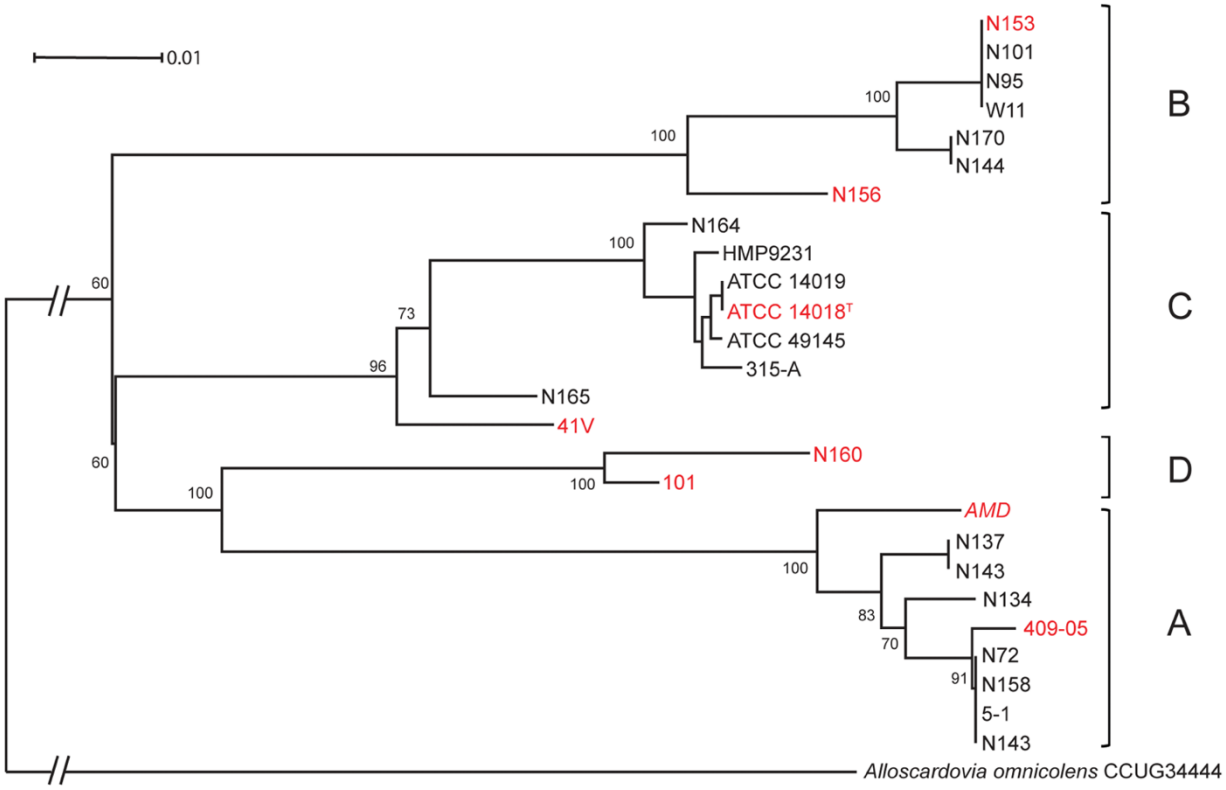

Figure 1.1. The four subgroups of *Gardnerella vaginalis* based on *cpn*60 sequences shown in a bootstrapped phylogenetic tree from previous work (Paramel Jayaprakash et al., 2012).

1.3.4 Association of *G. vaginalis* subgroups with BV and virulence factors

The recognition of distinct subgroups in *G. vaginalis* may provide at least a partial explanation for the phenomenon of asymptomatic BV and the inconsistent association of abundant *G. vaginalis* with symptoms of dysbiosis. The involvement that *G. vaginalis* subgroups have in BV is still being investigated, looking at what subgroups are associated with BV, and distinguishing between pathogenic and non-pathogenic subgroups.

Paramel Jayaprakash et al. (2012) analyzed *cpn*60 based microbiome profiles to determine the distribution of *G. vaginalis* subgroups in the vaginal microbiomes of African women diagnosed as BV positive (n=20), Intermediate (n=5) and Normal (n=19). All 44 samples contained at least one subgroup. The majority of samples (25/44) sequenced contained sequences from all four subgroups, while others contained different combinations of two or three subgroups. Subgroup B was significantly more abundant in BV compared to Normal microbiomes (Paramel Jayaprakash et al., 2012). Balashov et al. (2014) used clade (subgroup)-specific *G. vaginalis* PCR assays to examine 60 vaginal swab specimens and determine any correlation of subgroup and BV defined by Nugent scores and Amsel criteria. Clades 1 and 3 (subgroups C and D) were positively associated with high Nugent score 7-10 and positive Amsel's criteria. No association of clade 4 (subgroup A) and BV based on Nugent score or Amsel's criteria was observed. Clade 2 (subgroup B) was associated with intermediate Nugent scores (4-6) (Balashov et al., 2014). Multi-clade *G. vaginalis* communities were found to be positively associated with clinical BV symptoms and abnormal vaginal flora with Nugent scores 7-10 and negatively associated with Nugent scores 0-3 (Balashov et al., 2014). These results suggest an association that is stronger than that observed

for biotype and ARDRA genotype, however more work is needed with better defined subjects where symptoms/signs and microbiological assessment has been done.

The association of biofilm formation and *cpn*60 UT defined subgroups is not yet well characterized. In a study conducted by Paramel Jayaprakash, it was observed that biofilm formation of six subgroup A, two subgroup B, five subgroup C and two subgroup D *G. vaginalis* isolates varied (Paramel Jayaprakash et al., 2012). The growth and consistency was dependent on the type of media on which it was grown (brain heart infusion with glucose (BHIG) or ATCC #1685 broth) (Paramel Jayaprakash et al., 2012). Both subgroup B isolates formed biofilm on both types of media, however the biofilm formed in BHIG was more extensive than that formed in ATCC #1685 broth (Paramel Jayaprakash et al., 2012). Only one subgroup D isolate was tested and formed biofilm in BHIG but not ATCC #1685 media (Paramel Jayaprakash et al., 2012). In subgroups A and C at least one isolate failed to produce any visible biofilm in ATCC #1685 media (Paramel Jayaprakash et al., 2012). The variability of the biofilm formation of *G. vaginalis* subgroups on different media presents a challenge for using this trait in phenotypic characterization of the four subgroups. This also makes it difficult to determine what subgroups may be forming biofilm in the vaginal environment.

A study in 2016 looked at inherently metronidazole-resistant clades of *G. vaginalis*. Results of analysis of 88 strains for the minimum inhibitory concentration (MIC) showed that 43 % of clade 1 isolates, 7.1 % of clade 2 isolates, and 100 % of isolates in both clades 3 and 4 were metronidazole resistant (Schuyler et al., 2016). The resistance differed significantly between clades, being most frequently observed in clades 3 and 4 (Schuyler et al., 2016). Ahmed et al. (2012) demonstrated that phylogenetically clades 1 and 2 are more closely related to one another, and clades 3 and 4 are more related, which indicates that their similarity of metronidazole

resistance is most likely not due to horizontal gene transfer during treatment but common genetic ancestry (Schuyler et al., 2016). This may aid in explaining the common reocurrence of BV post-treatment, if a metronidazole susceptible strain (clade 1 or 2) is eliminated during the course of treatment but a resistant strain (clade 3 or 4) remains (Schuyler et al., 2016). The ability of different strains to cause disease may not be equal, as clade 4 is commonly not found in cases of BV, while clades 1 or 3 are often associated with BV (Balashov et al., 2014). More specific diagnostics such as clade/subgroups specific PCR for diagnosis may be benficial for treatment strategies of BV (Schuyler et al., 2016).

Sialidase activity is one of the most investigated virulence factors of *G. vaginalis*, and so determining its relationship to the four subgroups of *G. vaginalis* is of particular importance to understanding the roles of *G. vaginalis* subgroups in BV pathogenesis. The presence of sialidase in *G. vaginalis* has been characterized using a genotypic method screening for a putative sialidase gene (Pleckaityte et al., 2012; Santiago et al., 2011). Schellenberg et al. (2016b) examined a collection of 112 *G. vaginalis* isolates representing the four subgroups, and tested them for both sialidase activity and presence of the previously described sialidase gene. Subgroups B, C and D were gene positive but sialidase activity was only observed in subgroup B isolates and a few C isolates. Overall, sialidase activity did not correlate with the presence of the gene (Schellenberg et al. 2016b). These results led to two important conclusions: that extracellular sialidase activity is subgroup specific, and that another gene may be responsible for sialidase activity in positive isolates.

1.4 Sialidase

1.4.1 Bacterial sialidases

Sialidases (neuraminidases) are a large family of glycohydrolytic enzymes that break α-ketosidic linkages of terminal sialic acids from a variety of sialo-derivatives such as glycoproteins, glycolipids and oligosaccharides (Briselden et al., 1992; Giacopuzzi et al., 2012; Wiggins et al., 2001). Sialidases have undergone many studies since their discovery in the 1940s. Their occurrence in mammals, bacteria and viruses is widespread, and their catalytic domains are highly conserved (Corfield, 1992; Giacopuzzi et al., 2012). Sialidases in mammals occur in lysosomes or in the cytosol, and are associated with the plasma membrane (Johansson and Brett, 2003). Bacterial sialidase enzymes may be within the cytosol, secreted or cell-bound. In most cases, the secretion of sialidase is considered necessary to perform its physiological function (Corfield, 1992; Wiggins et al., 2001). It has been suggested that cell-bound sialidases may be a stored form of the enzyme before release, or that these enzymes may have a function in the periplasmic space in Gram negative bacteria (Guzman et al., 1990). As a result of the biological location of activity of sialidases, the pH optimum is typically within the range pH 5-7 (Corfield 1992). Microbial sialidases play many roles for the bacteria or viruses that produce it, including contributions to nutrition, or acting as virulence factors.

Sialidase activity has been observed in small amounts in non-pathogenic bacterial strains (Corfield 1992). These non-pathogenic bacteria may have cell-bound sialidase and thus exhibit lower levels of activity since the bacteria must be in contact with sialoglycan molecules, such as mucins, for the sialidase to cleave the substrate. Pathogenic bacteria tend to exhibit higher levels

of sialidase activity and the enzyme is secreted into the environment or anchored on the outside of the cell rather remaining within the cell (Corfield et al., 1992; Corfield, 1992).

Bacterial sialidases can act as virulence factors by aiding in attachment and invasion into mucosal surfaces found in the colon and vagina by degrading mucin molecules present in mucus. Sialidase has been observed to exert a toxic effect on host tissues, and interfere with immunologic and other defence mechanisms (Corfield 1992). The mammalian mucosal surface is rich in sialoglycans and mucus sialoglycoproteins that provide physical, immunological and bactericidal properties that protect the mucosa from pathogenic bacteria (Lewis and Lewis, 2012). Sialidases are able to cleave terminal sialic acids from mucins and allow bacterial cells to travel to the surface of epithelial cells and allow for adhesion and colonization. A number of immune proteins are encountered at mucosal surfaces such as secreted immunoglobulins (IgA, IgG), and mucosal epithelial cells also secrete surface glycoproteins that form a protective 'glycocalyx' vulnerable to degradation by sialidases (Lewis and Lewis, 2012).

1.4.2 Sialidases in BV

In the reproductive tract, women with BV have a polymicrobial community with a high production of sialidases and thus face a higher risk for infections and adverse pregnancy outcomes, as sialic acids are removed from complex N-glycans of secretory IgA, and the O-glycans of mammalian mucins rendering them ineffective (Lewis et al., 2012). This allows increased adhesive capability of some bacteria to epithelial cells, allowing them to proliferate and cause infection (Briselden et al., 1992). BV is characterized by the presence of sialidase activity due to the number of organisms that produce it in this dysbiosis. Bacteria involved in BV such as *Bacteroides*, *Prevotella* and *Mobiluncus* species, as well as one subgroup of *G. vaginalis* all produce sialidase.

The role of *G. vaginalis* and the presence of sialidase may help explain whether or not certain subgroups are pathogenic, or non-pathogenic. The lack of correlation of the presence of a putative sialidase gene with enzyme activity (Schellenberg et al., 2016b) and the absence of any direct evidence that this gene encodes a sialidase enzyme lead to some obvious research questions regarding genomic determinants of sialidase production in *G. vaginalis* subgroups.

2 Objectives

The multifactorial nature of bacterial vaginosis and its variety of clinical presentations create many challenges for clinicians in terms of diagnosis and treatment. Studies have shown that *G. vaginalis* is unable to cause BV alone, and works synergistically with other BV microbiota that then results in the observable microbiological and clinical signs of BV (Patterson et al., 2010). The characterization of *G. vaginalis* and determination of its role in BV development is a small piece of the puzzle that will hopefully shed light on how clinicians should approach asymptomatic BV, and intermediate vaginal microbiota, and whether or not treatment is beneficial. Characterizing *G. vaginalis* diversity in the context of the four *cpn*60-defined subgroups is a potential route to improved understanding of these issues.

The focus of this thesis is the characterization of sialidase genes and enzymatic activity in G. *vaginalis* subgroups through addressing these objectives:

- Identify sialidase genes and determine their distribution in a collection of 112 *G. vaginalis* isolates.
- Characterize the activity, pH optima and location of the two putative sialidase proteins.

3 Identification and distribution of sialidase genes in a collection of 112 *Gardnerella vaginalis* isolates

3.1 Abstract

Gardnerella vaginalis is a hallmark bacterium of a poorly understood dysbiosis known as bacterial vaginosis (BV) that is characterized by troubling symptoms and an overgrowth of anaerobic and facultative species of bacteria. BV contributes to poor reproductive outcomes such as preterm birth, and increased risk for transmission of sexually transmitted pathogens including HIV. *G. vaginalis* is found in asymptomatic, and symptomatic BV cases, making the role that *G. vaginalis* plays in the vaginal microbiome unknown. Virulence factors such as haemolysin, mucinase, lipase, prolidase and sialidase have all been reported characteristics of *G. vaginalis*. Historic literature has identified one sialidase gene (Gene 1) thought to be responsible for observed sialidase activity in some *G. vaginalis* isolates, however no studies have been done actually linking the gene with the production of a sialidase enzyme. Previous studies working with a culture collection of 112 representative isolates have shown based on the chaperonin-60 Universal Target (*cpn*60 UT) that *G. vaginalis* separates into four distinct subgroups, A, B, C and D. Subgroup B and a small number of subgroup C isolates had sialidase activity and it was clear that the presence of the previously described sialidase gene did not correlate with sialidase activity. Investigation of whole genome sequences resulted in discovery of a second putative sialidase gene (Gene 2). In the current study, further analysis of the predicted products of Gene 1 and Gene 2 indicated that Gene 1 has features characteristic of a cytosolic enzyme while Gene 2 appears to encode an extracellular sialidase. The distribution of Gene 2 in 112 isolates of *G. vaginalis* was determined using PCR. No subgroup (0/36) A isolates, 32/33 B isolates, 3/35 C isolates, and 0/8 D isolates were Gene 2 positive and the presence of Gene 2 correlated with the detection of sialidase activity. Phylogenetic analysis of Genes 1 and 2 showed that Gene 1 sequences clustered by subgroup, and Gene 2 was found only in subgroup B and a few subgroup C genomes. Gene 2 sequences do not cluster by

subgroup, indicating that subgroup C isolates may have obtained Gene 2 due to horizontal gene transfer from subgroup B. By characterizing distribution of sialidase activity in *G. vaginalis* subgroups we may better our understanding of its role in symptomatic and asymptomatic BV.

3.2 Background

Gardnerella vaginalis is a hallmark of Bacterial Vaginosis (BV), a common but poorly understood imbalance in the vaginal microbiome associated with a variety of negative health outcomes, such as preterm birth and increased risk of STI transmission (Larsson et al., 2005). Despite its strong association with BV, *G. vaginalis* it is also detected in healthy women who do not report symptoms of BV (Hickey and Forney, 2014). Using conventional microbiology and *cpn*60-based microbiome profiling it has been established that *G. vaginalis* comprises four genotypically and phenotypically distinct subgroups (A to D), which likely are separate species (Paramel Jayaprakash et al., 2012).

Sialidase activity is a key virulence factor that is commonly detected in *G. vaginalis*, though it is not common to all isolates (Hardy et al., 2017b; Santiago et al., 2011; Schellenberg et al., 2016b). Sialidases are produced by many bacterial species and can be involved in nutrient degradation as well as virulence (Corfield, 1992). These enzymes cleave terminal sialic acid residues off of sialogylcans including vaginal mucins and IgA, factors that are involved in forming an immunoprotective barrier in the vagina (Lewis et al., 2012). Losses of terminal sialic acids due to the action of sialidases make mucins vulnerable to other glycosidases. The loss of mucins in the vaginal environment increases the risk of infection and possibly contributes to adverse pregnancy outcomes such as chorioamnionitis and preterm birth (Cauci et al., 2005; Zhang et al., 2002).

Characterizing sialidase activity in *G. vaginalis* has involved application of qualitative activity assays, and a PCR assay designed to detect the presence of a putative sialidase gene originally identified in the whole genome sequence of *G. vaginalis* ATCC 14019 (sialidase A, locus tag HMPREF0421_20186 in Genbank Accession NC_014644) (Santiago et al., 2011). Santiago et al. (2011) genotyped 134 *G. vaginalis* isolates using amplified ribosomal DNA restriction analysis (ARDRA) and found three genotypes. These isolates underwent PCR screening for the sialidase gene, and all genotype 2 isolates were found to be negative, and genotypes 1 and 3 to be sialidase gene positive. When a sample of 33 isolates underwent a sialidase spot test for activity, genotypes 1 and 3 were sialidase positive and genotype 2 was sialidase negative (Santiago et al., 2011). Coincidentally these 33 isolates correlate presence of a sialidase gene with enzymatic activity (Santiago et al., 2011), however presence of this sialidase gene does not always correlate with activity (Schellenberg et al., 2016b). It is important to note that the *cpn*60 subgroup affiliations of the isolates characterized by Santiago et al. (2011) are not known or reported, and the use of ARDRA for genotyping may divide a single *cpn*60 subgroup into two genotypes. Using this PCR method appeared useful, however studies applying it often did not have phenotypic data to relate to their gene-based-findings. For example, Hardy et al. (2017b) examined the association between sialidase and biofilm formation, reporting presence of the sialidase gene but not activity. In 2016 Schellenberg et al. (2016b) characterized a collection of 112 *G. vaginalis* isolates that represent subgroups A, B, C, and D for sialidase activity and gene presence. Although subgroups B, C, and D are PCR positive for what has been assumed to be a sialidase gene (hereafter referred to as Gene 1) (Santiago et al., 2011), subgroup A, D and most C isolates are sialidase activity negative (Schellenberg et al., 2016b). Results of sialidase gene presence and activity assays would vary dramatically depending on the proportion of subgroups examined. For example, a collection

of subgroup C isolates would yield different results regarding sialidase activity than a collection with a high proportion of subgroup B isolates would. This may explain previous contradictory observations regarding correlation of the presence of Gene 1 and enzymatic activity.

Further investigation of *G. vaginalis* annotated whole genome sequences generated by our lab led to the discovery of another putative sialidase gene (Gene 2). Genes were initially identified based on gene annotation, by searching for the terms "BNR-Asp repeat", and "sialidase". No explicit link has yet been made between either Gene 1 or Gene 2 and enzyme activity. Characterizing sialidase activity and how the subgroups of *G. vaginalis* differ in pathogenicity will improve our understanding of the relationship between vaginal microbiome composition and health outcomes. The objective of this study was to determine the distribution of sialidase Gene 2 in 112 *G. vaginalis* isolates that represent all four subgroups in relation to their sialidase activity phenotypes.

3.3 Methods

3.3.1 Phylogenetic analysis and prediction of sialidase functional domains

The Integrated Microbial Genomes and Microbiome Samples database (IMG, https://img.jgi.doe.gov/cgi-bin/w/main.cgi accessed February 2016) was used to search 39 *G. vaginalis* whole genome sequences for putative sialidase genes (Gene 1 and Gene 2) based on annotation. Sialidase gene sequences were used for phylogenetic analysis. Forty-nine sialidase DNA sequences were aligned using ClustalW, trimmed to a uniform length of 2638 bp and used for tree building. The trimmed alignment went through bootstrapping (100 replicates), a distance matrix was calculated with dnadist and a tree was drawn using neighbor-joining in Mega v6 software.

Gene 1 and Gene 2 sequences were obtained from a previous whole genome sequence of *G. vaginalis* strain W11 (subgroup B) originally isolated from a Canadian woman. Inferred protein sequences encoded by Gene 1 and Gene 2 were analyzed using the Interpro Protein Sequence Analysis and Classification tool (EMBL-EBI, https://www.ebi.ac.uk/interpro/) to identify putative functional domains.

Bacterial sialidases most closely related to *G. vaginalis* Gene 1 and 2 products were identified by using predicted Gene 1 and Gene 2 product amino acid sequences from *G. vaginalis* W11 as queries in a BLASTp (Basic Local Alignment Search Tool, NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi) search of the NCBI non-redundant protein sequence database, and excluding results from *G. vaginalis*. There were four hits with the highest sequence similarity (88 to 90 % amino acid identity), and 20 hits ranging in similarity from 39 % to 48 % identity that were obtained, aligned using ClustalW and trimmed to a length of 532 aa and used for tree building. The trimmed alignment went through bootstrapping (100 replicates), a distance matrix was calculated with dnadist and a tree was drawn using neighbor-joining in Mega v6 software.

3.3.2 Bacterial culture and DNA extraction

Gardnerella vaginalis isolates were obtained from previous studies of women from Kenya, Canada and Belgium, as previously described (Schellenberg et al., 2016b). Freezer stocks in 4% (w/v) skim milk or NYC III medium (ATCC broth #1685) with 10% glycerol (v/v) were revived on Columbia 5% sheep's blood agar (CBA: BD Biosciences, Mississauga, ON) and incubated anaerobically at 37 °C using GasPak EZ sachets (BD 77 Biosciences, Mississauga, ON) in sealed jars for 48 hours. Isolates grown in liquid media were cultured anaerobically at 37 °C in NYC III

broth for 24 hours. Genomic DNA was purified from NYC III broth cultures using a modified salting-out procedure, as previously described (Martin-Platero et al., 2007).

3.3.3 PCR detection of sialidase Gene 2

Gene 1 prevalence in our collection of 112 isolates was determined previously (Schellenberg et al., 2016b). To screen isolates for the presence of Gene 2, PCR primers were designed based on multiple sequence alignments of 13 subgroup B and two subgroup C Gene 2 sequences obtained from the IMG database. Degenerate primers were designed to account for sequence variability within the gene sequence and to amplify a product of 375 bp in length (forward (JH0684): 5'-GTT GTA GAR CTT TCT GAT GG-3', reverse (JH0685): 5'-YRY TAT TAT CGC CCT CAT ATA-3'). Primers JH0684 and JH0685 were applied using conventional PCR to DNA extracts from 112 *G. vaginalis* isolates from Canada, Kenya and Belgium. PCR reactions contained 1× PCR Buffer (0.2 M Tris-HCl at pH 8.4, 0.5 M KCl), 2.5 μM $MgCl_2$, 0.40 μM dNTP, 0.20 μM forward primer, 0.20 μM reverse primer, 2 U *Taq* DNA Polymerase, ultrapure water and 2 μl of template DNA in a final volume of 50 μl. PCR reactions were conducted using the following thermocycling parameters: 94 °C for 3 minutes, 40 cycles of (94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds), 72 °C for 1 minute, hold at 20 °C. PCR products were visualized under UV light on a 1.0 % agarose gel containing ethidum bromide that was run at 100 V for 30 minutes.

3.4 Results

3.4.1 Identification of putative sialidase genes in *G. vaginalis* genomes

A search of 39 annotated *G. vaginalis* genome sequences (14, 11, 10, and 4 genomes from subgroups A, B, C, and D, respectively) with the functional terms "BNR/Asp-box repeat" and "sialidase" yielded 49 gene sequences. The BNR/Asp-box repeat is a repetitive sequence of amino acids present in some proteins such as neuraminidases/sialidases and a variety of glycosyl hydrolases. Phylogenetic analysis of the aligned DNA sequences resulted in a clear separation of two clusters with good bootstrap support (Figure 3.1), hereafter referred to as Gene 1 and Gene 2. Gene 1 corresponds to the putative sialidase gene targeted by previous PCR assays (Santiago et al., 2011) and is represented in this data set by *G. vaginalis* ATCC 14018 (IMG genome ID: 648276678). Gene 1 was present in genomes of all subgroup B, C and D genomes except for three subgroup C genomes (JCP7672, JCP7276 and 42431V) and one subgroup B genome (JCP8070). Pairwise DNA sequence identities among Gene 1 sequences ranged from 86 to 100 % identity over the length of the alignment. Sequences within the Gene 1 clade clustered according to *cpn*60 subgroup.

Gene 2 sequences, represented by 00703Bmash (IMG genome ID: 2600255001), were present in 10/11 subgroup B genomes and 3/10 subgroup C genomes. Sequence identities within this cluster ranged from 80 to 100 % identity, and unlike Gene 1, there was no apparent clustering of subgroup B and C sequences within the Gene 2 clade.

Similarities between the two orthologous sialidase gene clusters were much lower than within each clade. For example, the Gene 1 and Gene 2 sequences from W11 were only 49 %

identical at the DNA sequence level while Gene 1 and Gene 2 sequences in other strains were as low as 38 % identity.

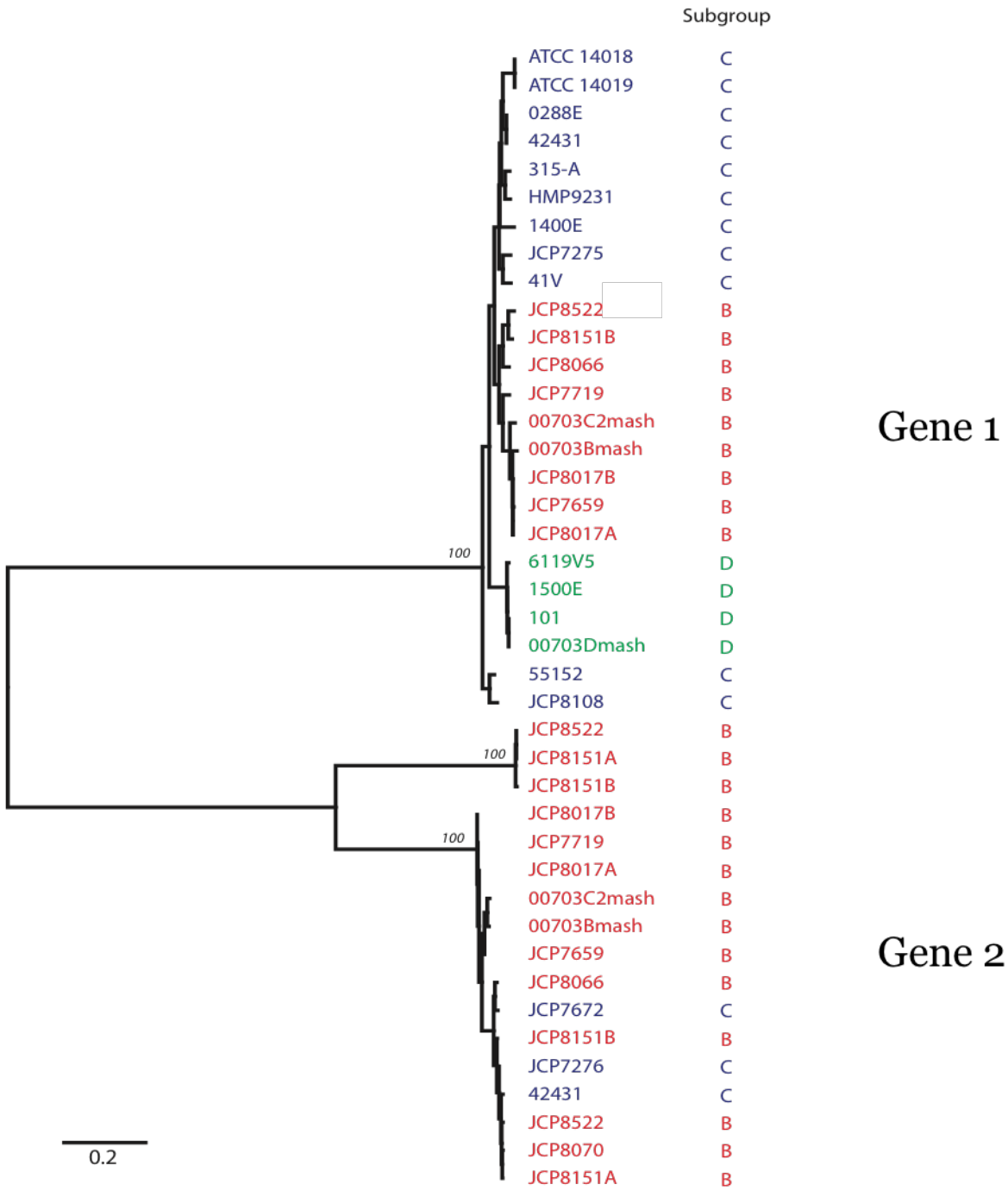

Figure 3.1. Phylogenetic relationships of Gene 1 and Gene 2 DNA sequences.

The tree is based on an alignment of 2638 bp. Bootstrap values (out of 100) are indicated at the nodes. Subgroup affiliation of each isolate is indicated based on *cpn*60 sequence.

3.4.2 Functional domain predictions for products of Gene 1 and Gene 2

To gain insight into the structures and possible functions of the products encoded by Gene 1 and Gene 2, representative sequences from strain W11 were subjected to functional domain prediction analysis using the Interpro Protein Sequence Analysis and Classification tool (EMBL-EBI, https://www.ebi.ac.uk/interpro/). The Gene 1 product showed an expected size of 99 kDa, with two sialidase domains and a lectin domain. Gene 2 predicted product showed an expected size of 89 kDa, with a sialidase domain, a non-cytoplasmic domain, a transmembrane domain, a cytoplasmic domain and an N-terminal signal peptide (Figure 3.2).

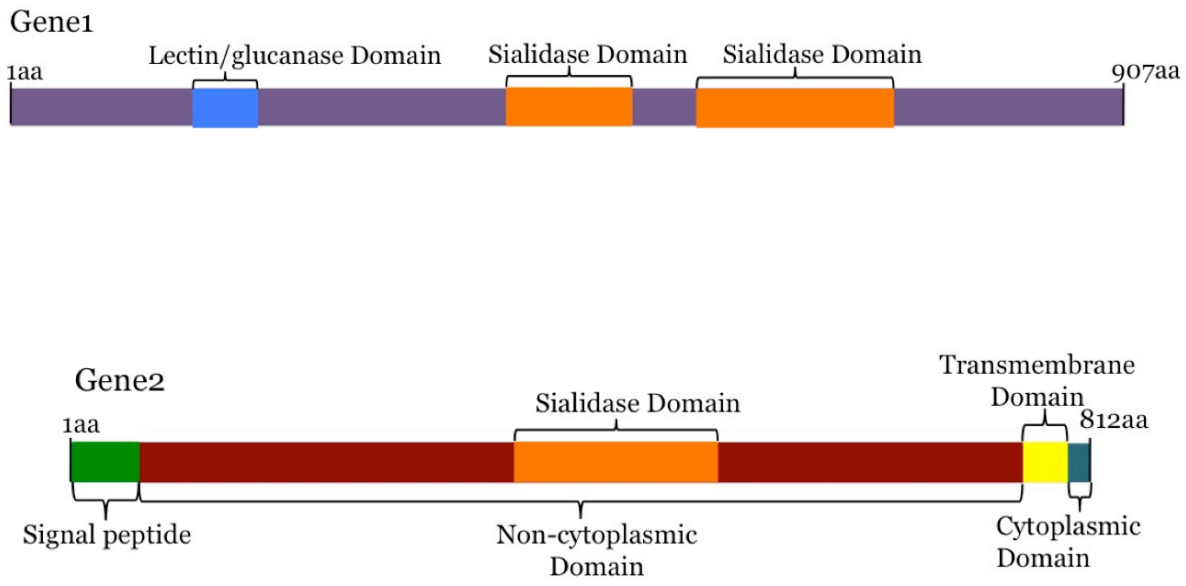

Figure 3.2. Predicted protein products of Gene 1 and 2.

Predicted domains of proteins encoded by Gene 1 and Gene 2 based on the Interpro Protein Sequence Analysis and Classification tool (EMBL-EBI, https://www.ebi.ac.uk/interpro/)

3.4.3 Phylogenetic relationships of *G. vaginalis* to other bacterial sialidases

The comparison of Gene 1 and 2 amino acid sequences to those in Genbank using BLASTp revealed a few highly similar hits from 88% to 96% identity and then a sharp drop off. A number of hits came up from *Bifidobacterium* and *Actinomyces* that were only 38% to 49% identical. Phylogenetic analysis of amino acid sequences of Gene 1 and 2 proteins revealed that the sialidase most similar to the Gene 1 protein was found in two strains of *Chlamydia trachomatis* H1IMS and SwabB1 (90% and 88% identity) and one strain of *Neisseria gonorrhoeae* SK708 (89% identity) (Figure 3.3). The most similar sialidase to Gene 2 protein was found in one strain of *Chlamydia trachomatis* H17IMS (96% identity). Further investigation of published whole genome sequences in the NCBI Refseq database from these organisms demonstrated that only 3/147 *C. trachomatis* and 1/438 *N. gonorrhoeae* genome sequences contained these highly similar sialidase proteins. Sialidases found in other genera varied in similarity ranging from 38% to 49% identity for both Gene 1 and 2 protein sequences. Sequences included in the phylogenetic tree were chosen to illustrate the diversity of organisms that have sialidases and compare similarity to protein sequences from Genes 1 and 2 (Figure 3.3).

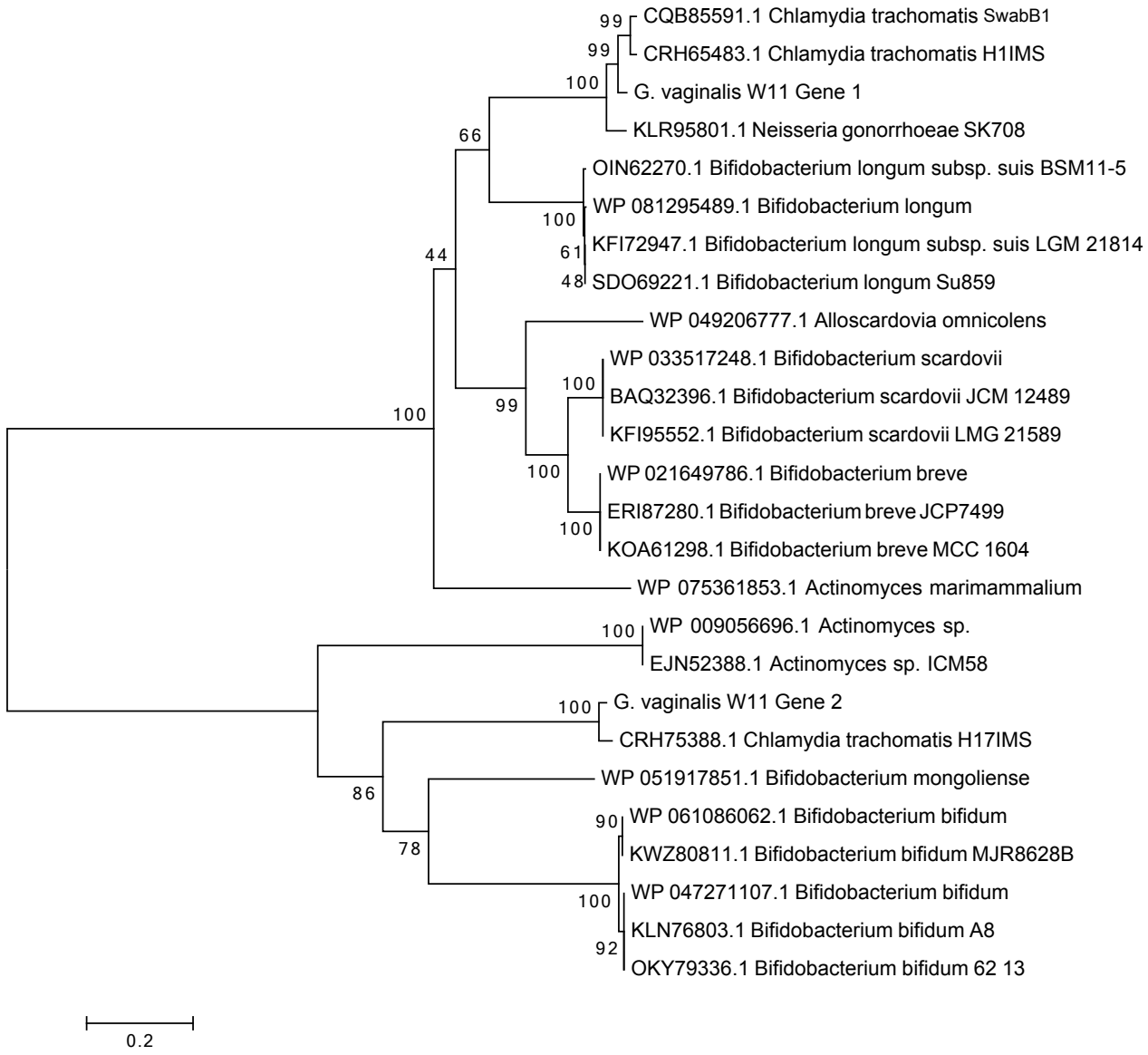

Figure 3.3. Phylogenetic relationships of *Gardnerella vaginalis* (strain W11) sialidase proteins with selected homologous sequences from other species.

Sequences were aligned using clustalw to a length of 532 aa. Each label shows the Genbank accession number, genus and species name followed by the strain name.

3.4.4 Screening of Gene 2 in 112 *Gardnerella vaginalis* isolates

Despite observing Gene 2 in whole genome sequences (Figure 3.1), it cannot be linked to sialidase activity, as there is no phenotypic data available for the isolates that were sequenced and submitted to IMG. In order to confirm that the presence of Gene 2 is correlated with sialidase activity, our collection of 112 *G. vaginalis* isolates for which phenotypic data was available was screened for the presence of Gene 2 using PCR primers JH0684/JH0685. All DNA extracts were tested by PCR for the chaperonin60 universal target (*cpn*60 UT) to confirm the quality of the DNA. The following PCR results were obtained: Subgroup A: 0/36 isolates positive for Gene 2, subgroup B: 32/33 isolates positive for Gene 2, subgroup C: 3/35 isolates positive for Gene 2, and subgroup D: 0/8 positive for Gene 2. Sialidase activity was observed exclusively in Gene 2 PCR positive isolates with the exception of one sialidase activity positive subgroup B isolate that was PCR negative (Figure 3.4).

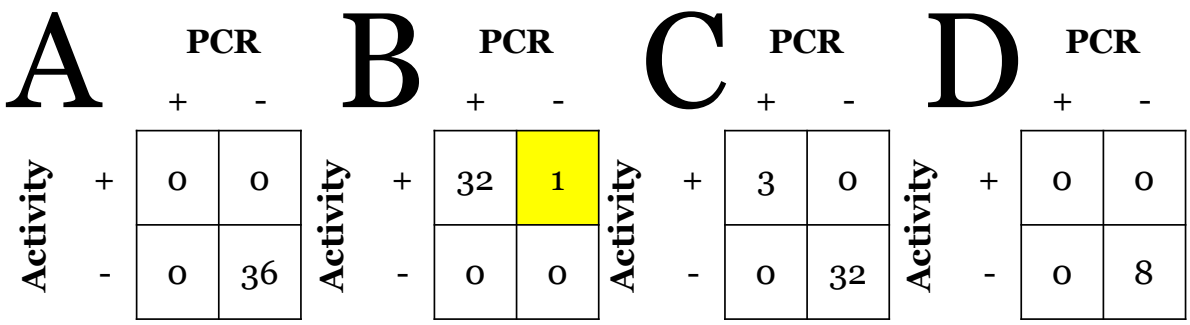

Figure 3.4. Gene 2 PCR and sialidase activity results for 112 *G. vaginalis* isolates.

A total of 112 *Gardnerella vaginalis* isolates in *cpn*60 subgroups A, B, C and D were examined. Yellow highlighted box emphasizes only one subgroup B isolate was PCR negative for Gene 2.

3.5 Discussion

Gardnerella vaginalis was first described in 1953 (Leopold, 1953) and identified as the causative agent of a newly described vaginitis, now known as BV (Gardner and Dukes, 1955). Over the years. studies have shown BV to be a complex, polymicrobial dysbiosis (Patterson et al., 2010). The role of *G. vaginalis* in BV is not understood, and its presence in healthy, asymptomatic women adds to the mystery (Dunkelberg, 1962; Schwebke, 2000). Characterization of this organism has revealed a vast amount of diversity, both genotypically and phenotypically. Sequencing analysis using the *cpn*60 universal target confidently shows the division of *G. vaginalis* into four distinct subgroups A, B, C and D (Paramel Jayaprakash et al., 2012), which can also be resolved based on whole genome sequencing (Ahmed et al., 2012; Schellenberg et al., 2016b). Phenotypic diversity has been illustrated with characterization of biotypes and presence of virulence factors in *G. vaginalis* (Piot et al., 1984; Pleckaityte et al., 2012). One such virulence factor of interest is sialidase, as activity and gene presence in *G. vaginalis* has been found to vary among isolates. A recent study shows that the presence of the previously described putative sialidase gene (Pleckaityte et al., 2012) does not correlate with sialidase activity and that only subgroup B and a few C isolates are sialidase activity positive (Schellenberg et al., 2016b). While subgroups B, C and D were all positive for this previously identified sialidase gene, and all A isolates were gene negative with one exception (Schellenberg et al., 2016b). These observations suggested that another gene might be responsible for observed sialidase activity, and led to the investigation for other putative sialidase genes in available whole genome sequences.

The search for sialidase-like genes (BNR-Asp box repeat/sialidase) in published *G. vaginalis* genomes resulted in the identification of 49 sialidase genes from 39 genomes. Phylogenetic analysis illustrated the presence of the previously described putative sialidase gene

(Gene 1) in subgroups B, C and D only. A second putative sialidase gene (Gene 2) was only present in subgroup B and three subgroup C genomes (Figure 3.1). Interestingly, different clustering patterns were observed within the Gene 1 and Gene 2 clades. Gene 1 sequences clustered according to *cpn*60 subgroup, indicating that a common ancestor of subgroups B, C and D had this gene, which was presumably lost by subgroup A at some point in its evolution. In contrast, the three subgroup C Gene 2 sequences do not cluster separately from the subgroup B sequences. Combined with the apparent rarity of Gene 2 in subgroup C, this pattern suggests that lateral gene transfer may have occurred at some point from subgroup B to some subgroup C isolates. Lateral gene transfer between *G. vaginalis* subgroups is not unexpected given that they inhabit a common microbiome and colonization with multiple subgroups is commonly observed in individual women (Albert et al., 2015). The absence of Gene 1 in three C genomes (JCP7672, JCP7276 and 42431V) and one B genome (JCP8070) may simply be due to the fact that these are draft genomes and that the complete genes were not assembled or annotated.

Gardnerella belongs to the Bifidobacteriaceae family and so it was not surprising that phylogenetic analysis (Figure 3.3) revealed related sequences from *Bifidobacterium longum*, *B. bifidum*, *B. scardovii*, *B. breve, Alloscardovia*, and *Actinomyces* although sequence identities were only 38% to 49% for both Gene 1 and Gene 2 protein sequences. Surprisingly, the most similar Gene 1 sialidase sequences were identified in *Chlamydia trachomatis* H1IMS and SwabB1 (88% and 90% identity) and *Neisserria gonorrhoeae* SK708 (89% identity). The highest similarity to the Gene 2 protein sequence was also found in *Chlamydia trachomatis* H17IMS (96% identity) (search conducted on 8th December, 2017). Further analysis of these genomes elucidates that these highly similar results from *N. gonorrhoeae* and *C. trachomatis* are from incomplete drafts. The likelihood that the presence of these *G. vaginalis*-like sialdiases is due to contaminants is quite

high. The *C. trachomatis* and *N. gonorrhoeae*. *G. vaginalis*-like sialidase sequences were detected in only 3/127 incomplete *C. trachomatis* genomes and 1/423 incomplete *N. gonorrhoeae* genomes which supports that this is due to contamination. However, if this is a real phenomenon it could occur due to sporadic lateral gene transfer as these organisms have a common environment.

The identified functional domains of the two proteins encoded by Gene 1 and 2 from representative subgroup B isolate W11 suggest that Gene 1 may encode an intracellular sialidase, while Gene 2 encodes an extracellular sialidase that is anchored to the cell membrane or secreted. In a recent study of *Bifidobacterium bifidum* a cell surface associated sialidase has been identified and tested for substrate degradation and how it aids in bacterial adhesion to host cells (Nishiyama et al., 2017). The *B. bifidum* sialidase and *G. vaginalis* Gene 2 sialidase were found to be 45% identical. The study of this extracellular sialidase may bring better understanding of the *G. vaginalis* Gene 2 sialidase. This may also contribute to the explanation as to why Gene 1 is observed in subgroup C and D isolates that do not have observable sialidase activity, while those isolates in subgroups B and C that have Gene 2 do have observable sialidase activity because that sialidase is outside the cell. The sialidase enzyme family is large and diverse, and includes enzymes that are involved in processing of nutrients as well as those that contribute to virulence through their action on host substrates like mucins and immunoglobulins (Corfield, 1992), so it is not surprising that *G. vaginalis* would have multiple sialidase enzymes with different biological roles.

Since results of the phylogenetic analysis only show presence of the sialidase gene, and cannot be connected with sialidase activity, PCR screening for the presence of Gene 2 in a collection of 112 *G. vaginalis* isolates representing the four subgroups was done, and compared with Gene 1 prevalence and sialidase activity data from a previous study (Schellenberg et al., 2016b). The summarized results in Figure 3.4 show that all but one of the sialidase activity positive

subgroup B and C isolates were Gene 2 positive. The single negative result from a subgroup B isolate may be due to diversity within this gene and simply the primers designed were not able to amplify Gene 2 in this particular isolate. These results illustrate a nearly perfect correlation of sialidase activity and Gene 2 presence, suggesting this gene may encode the enzyme responsible for observed extracellular sialidase activity.

3.6 Conclusions

Previous studies have left unresolved questions regarding the link between the presence of Gene 1 and sialidase activity of *G. vaginalis* isolates. This study shows the presence of a second putative sialidase gene (Gene 2), and the presence of this gene strongly correlates with sialidase activity. Taken together with analysis of predicted protein domains of the Gene 1 and Gene 2 products, our results support the hypothesis that Gene 1 encodes an intracellular enzyme, while Gene 2 encodes an extracellular enzyme responsible for the sialidase activity observed in assays of live *G. vaginalis* cultures. Our results also suggest that lateral gene transfer occasionally occurs resulting in sialidase activity positive subgroup C isolates. Lateral gene transfer may also have occurred between *G. vaginalis*, *Chlamydia trachomatis,* and *Neisserria gonorrhoeae*. Further investigation must be done to clone and express Genes 1 and 2 to illustrate that they do encode sialidase enzymes.

3.7 Transition Statement

Gardnerella vaginalis has genotypic and phenotypic diversity. By determining the distribution of sialidase genes in *G. vaginalis*, we may gain a better understanding of the role that *G. vaginalis* subgroups play in BV. Sialidase is a virulence factor that damages the immunoprotective barriers of the vaginal mucosa, which subsequently allows pathogens and other bacteria to flourish. Genetic determinants of sialidases in *G. vaginalis* have previously been unknown. We have demonstrated that the distribution of a previously described putative sialidase gene (Gene 1) does not correlate with observed sialidase positive phenotypes. Our analysis of whole genome sequences led to the identification of a second putative sialidase gene (Gene 2), and we have shown its presence correlates with sialidase positive phenotypes. However, neither gene has been shown to encode a sialidase enzyme.

4 Cloning and characterization of putative sialidases in a subgroup B *Gardnerella vaginalis* strain W11

4.1 Abstract

The elucidation of sialidase gene distribution in *G. vaginalis* subgroups and examination of the protein sequences encoded by Gene 1 and Gene 2 suggest that Gene 1 may encode an intracellular sialidase and Gene 2 may be responsible for extracellular sialidase activity. The goal of this study was to determine if these genes encode proteins with sialidase activity. Genes 1 and 2 were cloned into an expression vector (pQE-80L) with a 6-His tag for IPTG inducible expression and Nickel-NTA column purification. The protein from a Gene 1 clone was expressed using 0.5 mM IPTG and soluble protein was purified using Ni-NTA columns. Sialidase activity of purified Protein 1 was demonstrated using a qualitative spot test, and a quantitative kinetic fluorometric assay using 2'-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt hydrate (MUNANA) as substrate. The Activity of Protein 1 was measured over a range of pHs (3.5 to 7.5), and the highest activity was observed at pH 4.5 - 5.0. Initial attempts to express Gene 2 were unsuccessful due to a reading frame shift in a homopolymer region consisting of 10 cytosine residues 140 bp from the start of the gene. This shift may be due to slipped strand mispairing that causes variation in the number of repeated nucleotides. Examination of whole genome sequences and amplicon sequences from this gene region in other isolates revealed that this homopolymeric region is variable. To resolve this, a truncated version of Gene 2 was amplified and cloned, however expression of protein was again unsuccessful. Growth curves of *E. coli* containing Gene 2 clones under inducing conditions ruled out the possibility that the protein was toxic. The extracellular sialidase activity of *G. vaginalis* isolates was associated with the cell pellet while culture supernatants were sialidase negative. This indicates that the extracellular sialidase is anchored to the cell wall, consistent with the predicted protein product of Gene 2. Future work

could focus on the expression of the predicted sialidase domains found in Gene 2 to obtain a functional product as well as investigation of codon usage and other expression vectors.

4.2 Background

Genotypic and phenotypic diversity has frequently been observed in *G. vaginalis* since its discovery in 1953. There have been many methods implemented in typing *G. vaginalis*, for example biochemical diversity has been used for biotyping *G. vaginalis.* Benito et al. (1986) identified 8 biotypes using biochemical processes in *G. vaginalis*. A method to demonstrate genotypic diversity known as Amplified Ribosomal DNA Restriction Analysis (ARDRA) discriminates three genotypes based on the pattern of DNA fragments produced by restriction enzyme digestion of 16S rRNA gene PCR products (Ingianni et al., 1997). A more recent genotyping method is *cpn*60 UT based profiling which has provided resolution to *G. vaginalis* and shows it consists of four subgroups A-D (Paramel Jayaprakash et al., 2012). Biotyping and genotyping of *G. vaginalis* have been applied in attempts to understand why *G. vaginalis* can be isolated from asymptomatic women despite being considered a pathogenic entity associated with >98 % of BV cases (Cauci et al., 1996; Fredricks et al., 2007; Schwebke, 2000; Srinivasan et al., 2012). There are several virulence factors present in *G. vaginalis* that are ideal for colonizing the vaginal microbiome such as sialidase, lipase, haemolysin and biofilm formation, however not all strains of *G. vaginalis* have all of these virulence factors (Cauci et al., 2005; Cauci et al., 1996).

Sialidase is a virulence factor found in many pathogenic bacteria (e.g. *Vibrio cholerae*, *Clostridium perfringens*) and also plays a role in nutrition for some non-pathogenic bacteria (*Bifiobacterium longum* subsp. *infantis*) (Corfield, 1992). As a virulence factor, sialidase aids in breaking down immunoprotective barriers of mucosal membranes such as mucus. Mucus is a

major protective barrier in the reproductive tract that is largely composed of mucins. Mucin is a sialoglycan-rich molecule with terminal sialic acid residues, and the sialic acid bonds are commonly targeted by bacterial enzymes that cleave them, which aids in pathogen adherence (Lewis and Lewis, 2012). Mucus sialoglycoproteins have important immunological and physical properties that defend the underlying epithelial surfaces. Mucins provide substantial physical protection for vaginal epithelial cells as they form a lattice structure that excludes particles and bacteria from reaching the epithelium (Lewis and Lewis, 2012). The presence of bacteria such as *G. vaginalis* and other sialidase producing BV-associated organisms such as *Prevotella*, and *Bacteriodes* species, leads to a breakdown of these mucins. The degradation of mucin molecules allows other enzymes to cleave the subsequent linkages and as a result will leave open the epithelial layer for bacterial adhesion (Wiggins et al., 2001). Without this protective barrier, the risk for STI transmission and infections increases (Briselden et al., 1992; Lewis and Lewis, 2012; Lewis et al., 2013). It is important to note that healthy women's vaginal mucus does not contain sialidase, so presence of sialidase often is due to dysbiosis (Briselden et al., 1992).

Sialidase activity has been associated with bacterial vaginosis and adverse pregnancy outcomes (Cauci and Culhane, 2011). The presence of sialidase activity in *G. vaginalis* strains is thought to contribute to its pathogenicity and the role it plays in BV, although over the years, studies of *G. vaginalis* have shown that sialidase activity is not common to all strains. There have been several reports of *G. vaginalis* strains that were sialidase activity negative but PCR positive for a previously described sialidase gene, called Gene 1 throughout this project (Santiago et al., 2011; Schellenberg et al., 2016b). The presence of a second putative sialidase gene (Gene 2), however, does correlate to sialidase activity (Chapter 3). Over the years of studying *G. vaginalis* and sialidase no one has expressed and demonstrated the function of the protein encoded by Gene

1, or the more recently identified Gene 2. The objective of the current study was to express and characterize the putative sialidase genes present in *G. vaginalis* to determine if they do encode sialidase enzymes. This will provide more information to better our understanding of the role that different subgroups have in asymptomatic and symptomatic BV and how they may differ in pathogenicity.

4.3 Methods

4.3.1 PCR amplification of Gene 1 and 2

Primers to amplify the open reading frames of Gene 1 and Gene 2 from *G. vaginalis* isolate W11 were designed with restriction enzyme recognition sites for *Sph*I and *Kpn*I to facilitate insertion of the PCR products into expression vector pQE-80L. Primers for Gene 1 included forward primer JH0682 with *Sph*I restriction site underlined: 5'-TGC ATG CGA AGT CGT TCA ACG AA-3', reverse primer JH0683 with *Kpn*I underlined: 5'-CAG GTA CCC TAA TGT CTC TTC CA-3'. Primers for Gene 2 included forward primer JH0684 with *Sph*I restriction site underlined: 5'-GCA TGC ATT GGA ACA GCG ATA A-3', reverse primer JH0685 with *Kpn*I restriction site underlined: 5'-CAG GTA CCT TAA TAT TGC ATA TTT TTT AA-3' (Table 4.1; Figure 4.1). Reaction mixtures were based on manufacturer guidelines for using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Burlington, ON). Master mix contained final concentrations of 1× PCR buffer [60 mM Tris-SO_4 (pH 8.9), 18 mM $(NH_4)_2SO_4$], 2 mM $MgSO_4$, 0.20 mM dNTP mix, 0.20 μM forward primer, 0.20 μM reverse primer, template DNA, and 1.0 U proof-reading Platinum *Taq* DNA Polymerase High Fidelity. PCR reactions were performed using the following parameters: 94 °C: 2 minutes, (94 °C 30 seconds, 58 °C 30 seconds, 72 °C 3 minutes)

for 40 cycles, 72 °C 10 minutes, hold at 20°C. PCR products were resolved on a 0.8% agarose gel and purified with a gel-extraction kit (BioBasic, Markham, ON).

Table 4.1 Primers used in this study.

Gene Target	Application	Primer Name	Primer Sequence (5`-3`)	Annealing temperature	Product size	Reference
Gene 1	Cloning	JH0682	TGC ATG CGA ACG TCG TTC AAC GAA G	58.1°C	2736	This study
		JH0683	CAG GTA CCC TAA TGT CTC TTC CAT TTT GGC T			
Gene 2	Cloning	JH0684	GCA TGC ATT GGA ACA GCG CAT AAA G	58.1°C	2450	This study
		JH0685	CAG GTA CCT TAA TAT TGC ATAT TTT TTA A			
Gene 2	Truncated Gene 2 for cloning	JH0762	TTG CAT GCC AAA CTG AAC CAG TAA	58.1°C	2338	This study
Gene 1	Internal sequencing	JH0686	AAA CGG TGC GAA AGA AAT TG	N/A	N/A	This study
Gene 1	Internal sequencing	JH0687	ACG CTA ATC GAC GAC GAA AC			
Gene 1	Internal sequencing	JH0688	TGG CAA AAA CCT TGG AAA AC	N/A	N/A	This study
Gene 1	Internal sequencing	JH0689	GTT AAG GAG CCG TGG ATG AG			
Gene 2	Internal sequencing	JH0690	GCA CGC AAG GAT TCC ATA TAA	N/A	N/A	This study
Gene 2	Internal sequencing	JH0691	CGA TGG TGG AGT ATG GGA TT			
Gene 2	Internal sequencing	JH0692	TGA GAC ACT GCC AGA TCC AG	N/A	N/A	This study
Gene 2	Internal sequencing	JH0693	TGA AGT GCC AAA AAC AGC AA			
pQE-80L	Type III/IV pQE sequencing	JH0694	CGG ATA ACA ATT TCA CAC AG	N/A	N/A	Qiagen
		JH0695	TGA AGT GCC AAA AAC AGC AA			
Gene 2	Degenerate short amplicon	JH0720	GTT GTA GAR CTT TCT GAT GG	55°C	380	This study
		JH0721	YRY TAT TAT CGC CCT CAT ATA			
Gene 1	Internal sequencing	JH0760	CAA AAT CGC TCG GGA TGT GT	N/A	N/A	This study
Gene 2	Internal sequencing	JH0761	TTA GCG TAT CCC CAA CTC GA	N/A	N/A	This study
Gene 2	Internal sequencing	JH0765	TAA ATC ATC CGT ACG TGT AAT CG	N/A	N/A	This study

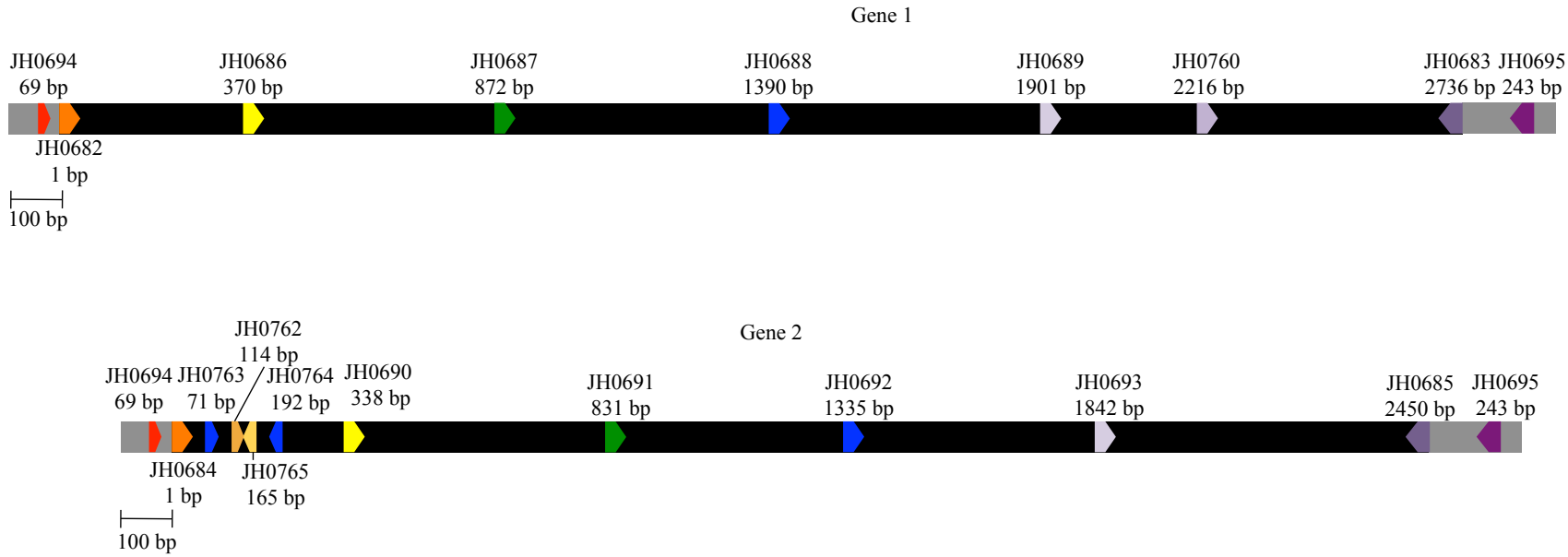

Figure 4.1 Primer map.

Location and direction (forward or reverse) of primers for PCR amplification and sequencing of Gene 1 and 2 that were used throughout the course of this study. The primer name and distance from the 5' end where the primer starts is listed above the arrow indicating direction. The black indicates the length of the gene and grey indicates the vector on either side.

4.3.2 Restriction Enzyme Digestion

Gel-purified products of Gene 1 and Gene 2 and expression vector pQE-80L underwent digestion with restriction enzymes *Kpn*I and *Sph*I. Digestion occurred under the following conditions, 0.5 µg of product DNA or vector, 10 U of *Kpn*I, 10 U of *Sph*I, 5 µl of NEBuffer 1.1 and molecular grade water for a final volume of 50 µl. Reactions were incubated for 1 hour at 37 °C, and inactivated at 65 °C for 20 minutes, and purified using a PCR clean up kit (BioBasic, Markham, ON). DNA concentration and quality was measured using a spectrophotometer.

4.3.3 Ligation of Gene 1 and Gene 2 into pQE-80L

A molar ratio of insert DNA to vector DNA of 3:1 was used for ligation reactions, which included 10 µl of 2× ligase buffer [60 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% (v/v) polyethylene glycol (MW8000, ACS Grade)], 6 U of T4 DNA ligase, and molecular grade water to a total volume of 20 µl. Reactions were incubated overnight at 4 °C.

The transformation of One Shot Top10 Chemically Competent *E. coli* Cells (Invitrogen, Burlington, ON) with ligation reaction products was done using the methods supplied by the manufacturer. Ligation reaction products (1-5 µl) were added directly to 50 µl of OneShot cell suspension and mixed gently, vials were then incubated on ice for 30 minutes and subsequently heat shocked at 42 °C for 30 seconds. A plasmid vector provided with the kit was used as a positive control in a separate transformation reaction. Pre-warmed SOC media (250 µl) was added to each vial and incubated for 1 hour at 37 °C with shaking at 225 rpm. Transformed cultures (20 µl, 55 µl, 75 µl or 100 µl) were then plated onto LB media plates containing 100 µg/ml ampicillin and

incubated overnight at 37 °C. Colonies were randomly selected and sub-cultured onto an LB media patch plate and underwent a colony PCR with gene specific primers (JH0682/683 and JH0684/685) (Table 4.1 and Figure 4.1). Positive clones were then cultured in 3 ml of LB broth containing 100 µg/ml ampicillin overnight at 37 °C and plasmids were purified from overnight cultures using a commercial kit (Plasmid DNA miniprep kit, BioBasic, Markham, ON). The presence of inserts was confirmed by gene specific PCR (JH0682/683 and JH0684/685) and restriction enzyme digestion with *Kpn*I and *Sph*I and agarose gel electrophoresis. Candidate clones were sequenced using primers JH0682, JH0683, JH0686-689, and JH0761 for Gene 1, and JH0684, JH0685, JH0690-693, JH0760, JH0762 and JH0765 for Gene 2, pQE-80L primers JH0694 and JH0695 described in Table 4.1 with locations and direction of primers shown in Figure 4.1. Sequencing was performed by Macrogen (Korea). Positive transformants were grown in LB broth with 100 µg/ml ampicillin and stored at -80°C in LB broth with 100 µg/ml ampicillin and 10% (v/v) gycerol. For the construction of truncated Gene 2 amplicon primer set JH0684 and JH0762 were used under the PCR conditions as described above.

4.3.4 Protein expression and purification

4.3.4.1 Initial protein expression test

Transformants were cultured in 3 ml of LB broth containing 100 μg/ml ampicillin and incubated at 37 °C overnight. Overnight culture (1 ml) was then used to inoculate 5 ml of prewarmed LB broth containing 100 μg/ml ampicillin, which was then incubated at 37 °C until an optical density measured at 600 nm (OD_{600}) of 0.5-0.7 was reached. OD_{600} was measured using a Nanodrop Spectrophotometer 2000. Protein expression was induced with the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and incubated for 4 hours with shaking at 37 °C. Induced cultures were centrifuged at 13,000 × g for 5 minutes and the resulting cell pellet was re-suspended in 200 μl SDS sample buffer (62.5 mM 1.0 M Tris (pH 6.8), 0.465% (w/v) of 10% SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.00250% (v/v) of 2.5% bromophenol blue). Samples were boiled for 2 minutes and then sonicated for 3 seconds. The resulting viscous lysate was clarified by centrifugation for 5 minutes at 13,000 × g. The samples were chilled on ice during all manipulations.

Lysates were then evaluated using SDS-PAGE. Samples (IPTG induced and non-induced controls) were loaded onto an 8% polyacrylamide gel to visualize the protein profile of the crude lysate by staining with Coomassie blue stain to determine if there was protein expressed from the cloned gene. The SDS-PAGE was run at 150 V for approximately 2 hours, or until the SDS sample buffer ran off the bottom of the gel. The gels were placed in Coomassie stain for 1 hour on a rocking platform and then placed in Coomassie destain and left on the rocking platform overnight.

4.3.4.2 Determination of optimal conditions for expression of Protein 2

The expression of Protein 2 was conducted as described above with the addition of determining an optimal incubation temperature, time and IPTG concentration. A Gene 1 clone was used as a control for successful protein expression. IPTG concentrations of 0.5 mM, 1.0 mM and 5.0 mM were tested under 37 °C for 4 hours, 30 °C for 6 hours and 24 °C overnight.

4.3.4.3 Large-scale purification of Protein 1

A 50 ml LB plus ampicillin culture of *E. coli* containing pQE-80L with Gene 1 was induced with 0.5 mM IPTG at OD_{600} 0.5-0.7, followed by incubation at 37 °C with shaking (225 rpm) for 4 hours. Cells were pelleted by centrifuging for 15 minutes at 5,000 × g, and re-suspended in 5 ml lysis buffer (25 mM Tris-HCl, 0.5 mM NaCl, 5% (v/v) glycerol), and sonicated with ten 15 second bursts with 1 minute rest on ice in between. Sonicated lysates were then centrifuged at 37000 × g for 30 minutes to pellet cell debris, and the clarified supernatant was added to a 10 ml His-Gravitrap Nickel column (GE Healthcare UK Limited, Buckinghamshire, England) that was pre-equilibrated with 15 column volumes (CV) lysis buffer. Columns were washed twice with 10 CV washing buffer (25 mM Tris-HCl (pH 7.4), 0.5 mM NaCl, 50 mM imidazole, 5% glycerol). Bound protein was eluted by running 15 CV of elution buffer (25 mM Tris-HCl (pH 7.4), 0.5 mM NaCl, 500mM imidazole, 5% glycerol) through the column and eluate fractions of 1.5 ml were collected into 1.5 ml microcentrifuge tubes. Purified protein was confirmed by SDS-PAGE, including a pre-stained protein ladder (Fisher Bioreagents, Fisher Scientific, Ottawa, ON). Purified protein fractions were put into dialysis tubing that was clamped at both ends, and suspended in 1 L of dialysis buffer (30 % (v/v) glycerol, 25 mM Tris-HCl, 150 mM NaCl, pH 8.0) and incubated overnight at 4 °C. Protein concentration was determined by Nanodrop spectrophotometer.

Dialysed protein solutions were transferred into micro-centrifuge tubes in 20 µl aliquots, and flash frozen in liquid nitrogen before being transferred to -80 °C freezer for storage.

4.3.4.4 Sialidase activity assays

A qualitative filter paper spot test was used to detect sialidase enzyme activity of *G. vaginalis* isolates, as previously described (Moncla and Braham, 1989; Pleckaityte et al., 2012; Santiago et al., 2011). Subsequently, a kinetic assay using quantitative fluorometry was applied to measure sialidase activity over time (Lewis et al., 2013). Both assays use the fluorogenic substrate 2'-(4-methylumbelliferyl)-α–D-N-acetylneuraminic acid sodium salt hydrate (MUNANA) (Sigma-Aldrich Canada, Oakville, ON) dissolved in water to a concentration of 300 µM (0.015% w/v) and aliquots stored at -20 °C. Prior to the assay, aliquots of substrate were thawed and diluted with 1.0 M sodium acetate (pH 5.8) to a final concentration of 243 µM.

To assess sialidase activity of whole bacteria, 10 µl of substrate was applied to Whatman qualitative filter paper Grade 1 circles (made using a regular hole punch) placed in a Petri dish and 10 µl bacterial culture was added to each circle and incubated for 30 minutes in the dark at 37 °C. Sialidase activity was detected by visualizing and photographing filter paper circles under UV light. For the kinetic, quantitative assay, 90 µl of substrate was combined with 10 µl of culture in duplicate wells of opaque 96-well plates, prior to measuring RFU over time in an FLx800 fluorometer (BioTek Inc., Winooski, VT). Readings were taken over a 30-minute period at 2-minute intervals after 6 seconds shaking at 30°C. The rate of substrate conversion in positive samples was expressed as the increase in RFU over time.

To measure sialidase activity of purified proteins and determine the effects of pH on activity, the kinetic assay was used as described above with buffers of different pH. MUNANA

substrate was prepared with different buffers to obtain a range of pH from 3.5 to 7.5 with 0.5 increments. Stock solutions of 1.0 M mono- and di-basic sodium phosphate were prepared, and mixed in different ratios to obtain the approximate desired pH with a total volume of 100 ml. Buffers were finely adjusted with sodium hydroxide or hydrochloric acid to obtain pH 6.0 to 7.5. A stock solution of 1.0 M sodium acetate and sodium phosphate buffers was prepared and 50 ml was aliquoted into bottles. The pH was adjusted with 5.0 N hydrochloric acid to obtain a range from pH 3.5 to 6.0. All sodium acetate and phosphate buffers were autoclaved, and stored at room temperature.

4.4 Results

4.4.1 Amplification and cloning of Gene 1

Gene 1 (2.7 kb) was amplified using proof-reading *Taq* polymerase (Figure 4.2A) for insertion into vector pQE-80L. Candidate clones were identified by screening colonies with gene-specific PCR (Figure 4.2B) as well as with restriction digestion (Figure 4.2C). In-frame fusion with the 6-His tag and accurate amplification of Gene 1 was confirmed by sequencing of the insert and comparison to previously determined sequence of *G. vaginalis* W11 Gene 1.

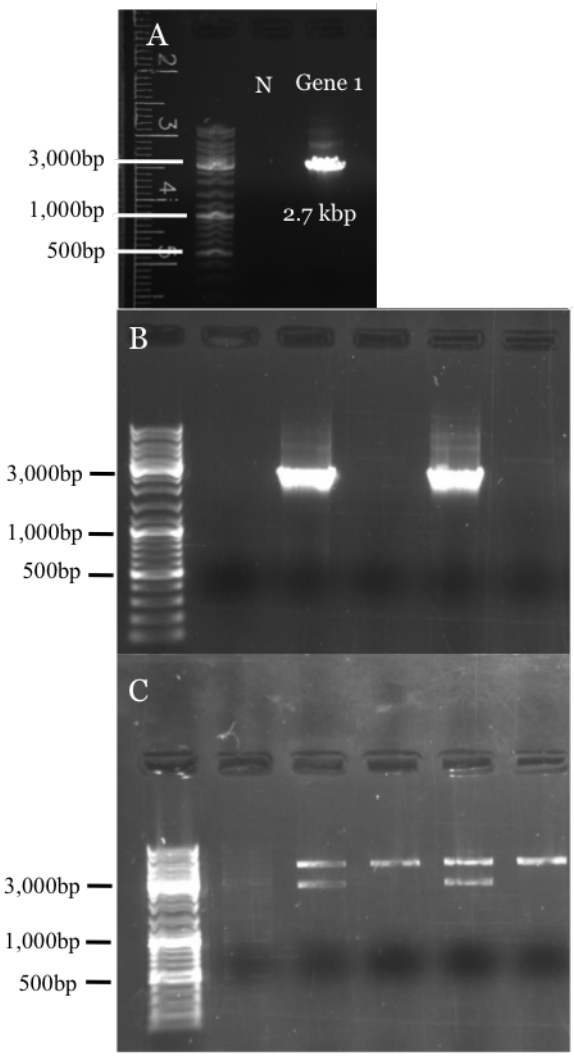

Figure 4.2. PCR amplification of Gene 1 and example of screening of candidate clones by restriction digestion.

A) Gene 1 amplicon was obtained using proofreading Platinum *Taq* DNA Polymerase from subgroup B *Gardnerella vaginalis* strain W11. Products were then digested with restriction enzymes *Kpn*I and *Sph*I and ligated into expression vector pQE-80L. B) PCR amplification of Gene 1 from individual *E. coli* colonies after transformation with pQE-80L containing Gene 1. Two samples are Gene 1 positive and three samples are negative for Gene 1. C) Results of digestion of candidate Gene 1 clones with *Kpn*I and *Sph*I. The resulting two bands are pQE80L (4.7 kb) and Gene 1 (2.7 kb). Lanes that show a single 4.7 kb band that is only vector, a negative clone. The empty lane did not contain the vector or Gene 1.

4.4.2 Expression of Protein from a Gene 1 clone

Initial expression tests with Gene 1 clones were conducted as described above with 1.0 mM IPTG for induction (Figure 4.3). Protein of the expected size for Gene 1 at 99 kDa was observed on SDS PAGE of cell lysates (Figure 4.3). Since Gene 1 successfully expressed protein on a small scale, it was then performed in a larger scale (50 ml volume of culture). Protein 1 was purified with Ni-NTA columns and found to be soluble (Figure 4.4). The purified fractions 1 and 2 underwent dialysis, were aliquoted and flash frozen in liquid Nitrogen and stored at -80 ° C for future enzymatic tests. The final concentration of Protein 1 was 460 ng/µl.

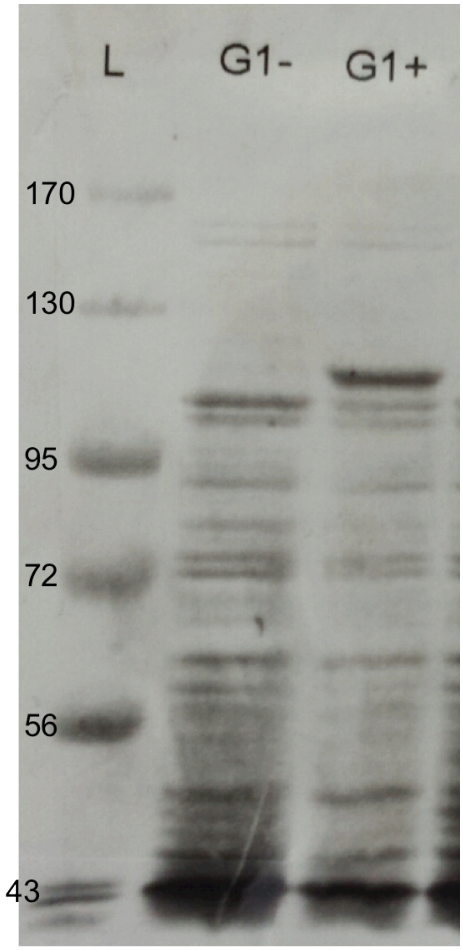

Figure 4.3. Protein profiles of cell lysates of *E. coli* containing pQE-80L plus Gene 1.

A candidate clone containing pQE-80L with Gene 1 (G1) was grown under inducing conditions for protein expression, and one sample was grown under regular conditions with no expression induction. Lane 2 containing Gene 1 shows a dark band of the expected size 99kDa that is not present in lane 1 on SDS PAGE**.**

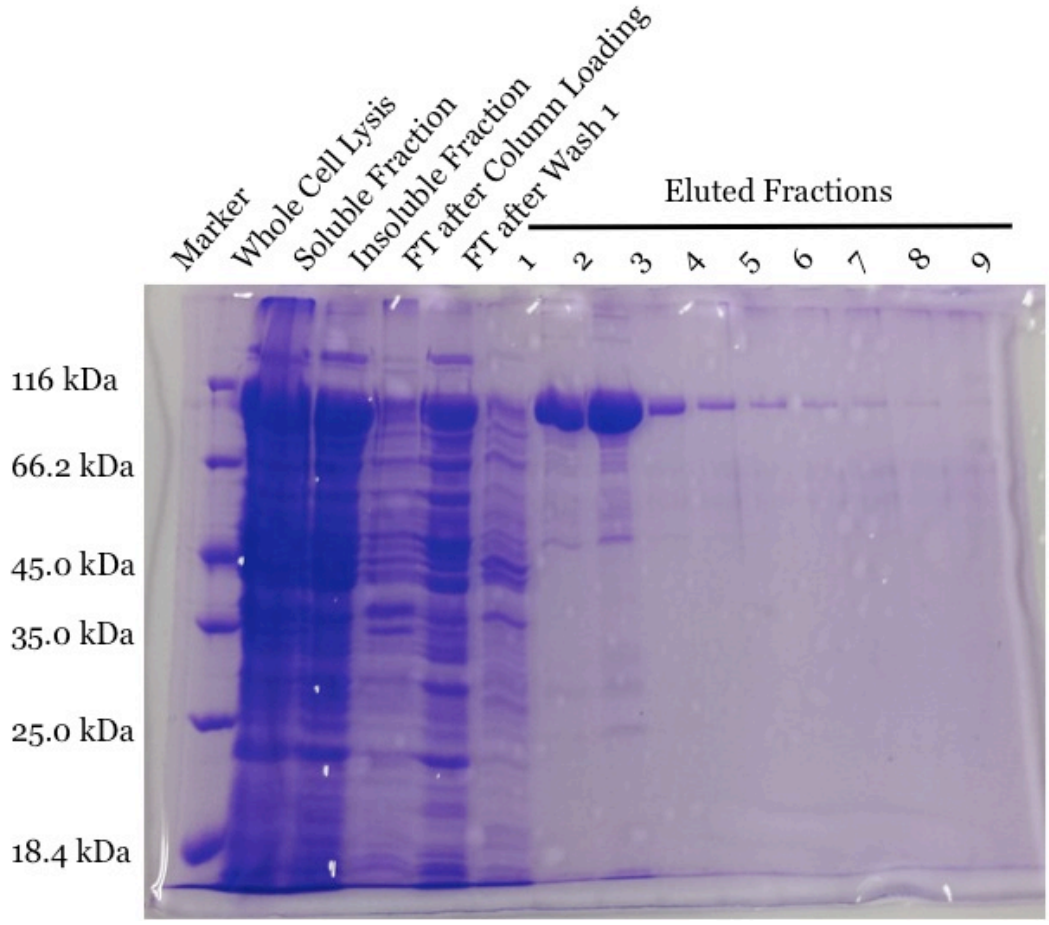

Figure 4.4 Purification process on SDS PAGE.

A fraction at each stage of the purification process of Protein 1 using Ni-NTA columns was run on SDS PAGE. Fractions 2 and 3 were flash frozen in buffer containing 30% v/v glycerol for future assays (FT = flow through).

4.4.3 Sialidase activity of Protein 1

Following confirmation of sialidase activity of purified Protein 1 using the filter spot test, activity of purified Protein 1 was tested in the kinetic assay at four concentrations: 460 nM, 370 nM 230 nM and 92 nM (Figure 4.5). Protein 1 has sialidase activity as it successfully cleaved MUNANA resulting in fluorescence. As expected rate of activity and enzyme concentration shows a positive linear relationship, although the slope was lower than expected (doubling enzyme concentration should result in doubling of rate) (Figure 4.5).

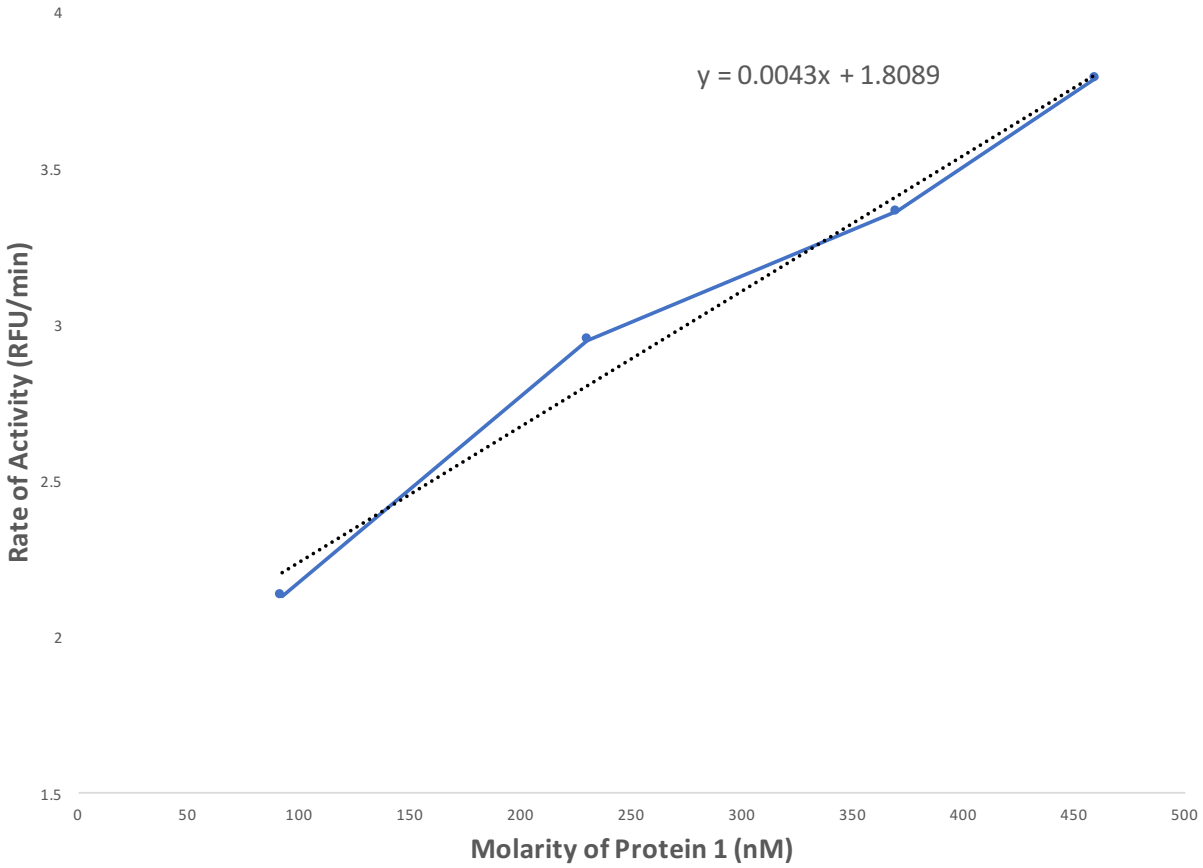

Figure 4.5. Relationship of Protein 1 concentration and sialidase activity.

Activity was tested using an FLx800 fluorometer and the fluorogenic substrate 2'-(4-methylumbelliferyl)-α–D-N-acetylneuraminic acid sodium salt hydrate (MUNANA). This illustrates that with increasing molarities of Protein 1, there is an increase in the rate of activity measured and has a positive linear relationship (the dotted line represents the line of best fit).

4.4.4 Determination of pH optimum of Protein 1

Activity of Protein 1 was measured at pH 3.5 to pH 7.5 with pH 0.5 intervals in triplicate with two technical replicates (Figure 4.6). Data points plotted in Figure 4.6 each represent the mean reading of a total of six replicates. The highest activity observed was at pH 4.5 - 5.0.

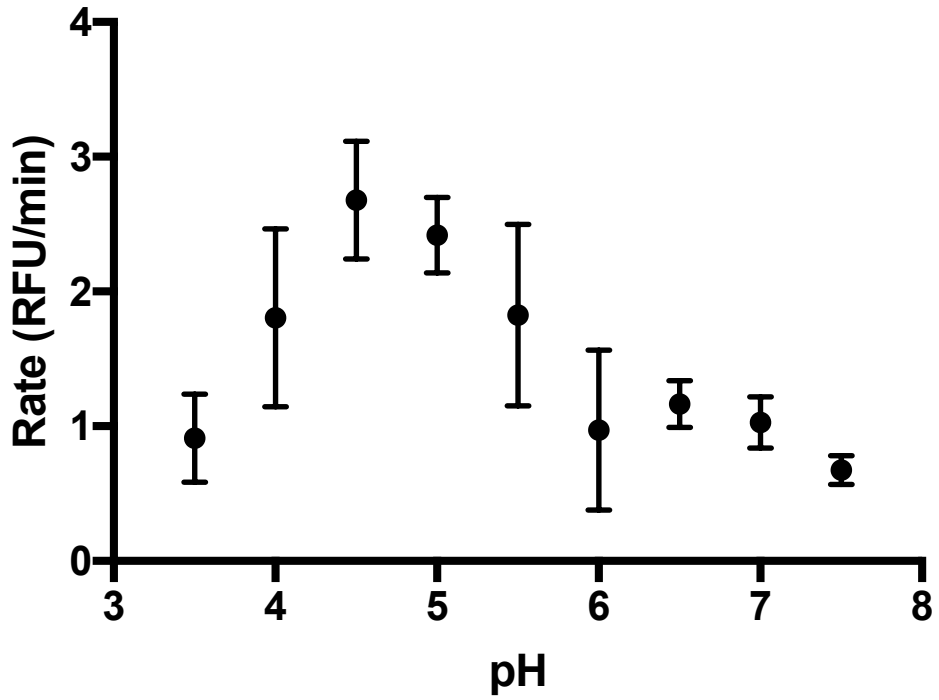

Figure 4.6. pH profile of Protein 1.

Average activity rate of Protein 1 from two technical replicates with a total of six readings for each pH tested. Error bars indicate standard deviation

4.4.5 Amplification and cloning of Gene 2

Gene 2 (2.4 kb) was amplified using proof-reading *Taq* polymerase (Figure 4.2) for insertion into vector pQE-80L. Sequencing of the construct indicated that the initially selected clone contained a reading frame shift resulting in a premature stop codon. This sequence is discussed in more detail below. The cloning process was repeated and an in-frame clone was obtained.

4.4.6 Expression of Protein from Gene 2 clone

Initial expression tests with the Gene 2 clone was conducted as described above with 1 mM IPTG for induction. Protein of the expected size for Gene 2 at 89 kDa was not observed on SDS PAGE of whole cell lysate. This led to investigation of the effect of IPTG concentration, incubation temperature and incubation time. IPTG concentrations 0.5 mM, 1.0 mM and 5.0 mM were tested with incubation at 37 °C for 4 hours, 30 °C for 6 hours or 24 °C overnight. Expression testing was conducted using pQE-80L+Gene 1 as a positive control. While Gene 1 showed a response to increasing the concentration of IPTG, observed on the SDS PAGE as a darker band at the expected protein size, no expression was observed from the Gene 2 clone (Figure 4.7).

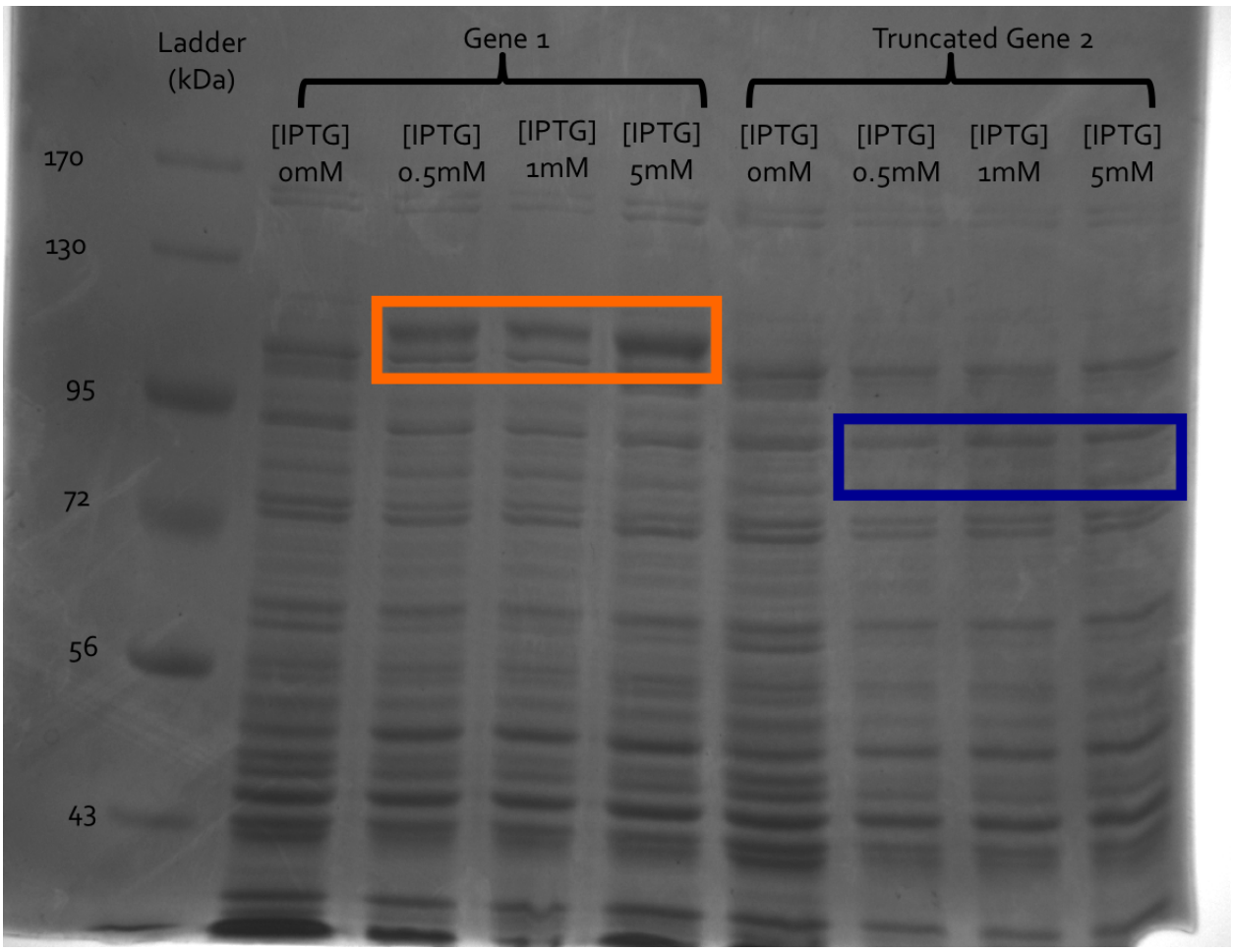

Figure 4.7. Effects of increasing IPTG concentration on expression of Gene 1 and Gene 2.

Expected product sizes were 99kDa for Gene 1 and 89kDa for Gene 2. Highlighted in the orange box is protein of the expected size expressed by Gene 1 clones. The Gene 2 clone did not express protein of the expected size highlighted in the blue box.

4.4.7 Investigation of the Gene 2 DNA sequence

Examination of the assembled Gene 2 sequence from the initial clone revealed a cytosine (C) homopolymer region at 141 to 151 bp that caused a frame-shift and premature stop codon (Figure 4.8). When Gene 2 sequences from other subgroup B *G. vaginalis* isolates were compared to the Gene 2 sequence extracted from the W11 whole genome sequence, the Gene 2 sequence from the clone in question, and a Gene 2 PCR amplicon from W11 DNA it was found that this homopolymer region varied from 12 C residues in the Gene 2 sequence from the W11 genome to 9 C residues in *G. vaginalis* N101 (Figure 4.8). There were ten C residues in the Gene 2 clone, which resulted in the frame shift that prevented protein expression.

Observation of a variable length homopolymer region could indicate that Gene 2 is subject to a slipped-strand mispairing mechanism. This resulted in an attempt to amplify a truncated Gene 2 product that excluded the homopolymer region that was cloned and tested for protein expression. A forward primer was designed (JH0762) downstream from the homopolymer region at 114 bp from the start of Gene 2 (Figure 4.1). No protein was expressed from this clone (data not shown), which led to the idea that the product may be toxic, killing the cells before there is sufficient expression to see protein on the SDS PAGE. A toxicity test was done in the form of a growth curve that included Gene 1 clone, empty pQE-80L vector, full length Gene 2 clone and truncated Gene 2 clone, all in *E. coli* grown under inducing conditions. Growth was measured using OD_{600} at 0, 1, 3 and 6 hours incubation (Figure 4.9). All cultures underwent log phase growth, similar growth curves were obtained in all cases, and there was no difference between empty vector and the full-length Gene 2 clone.

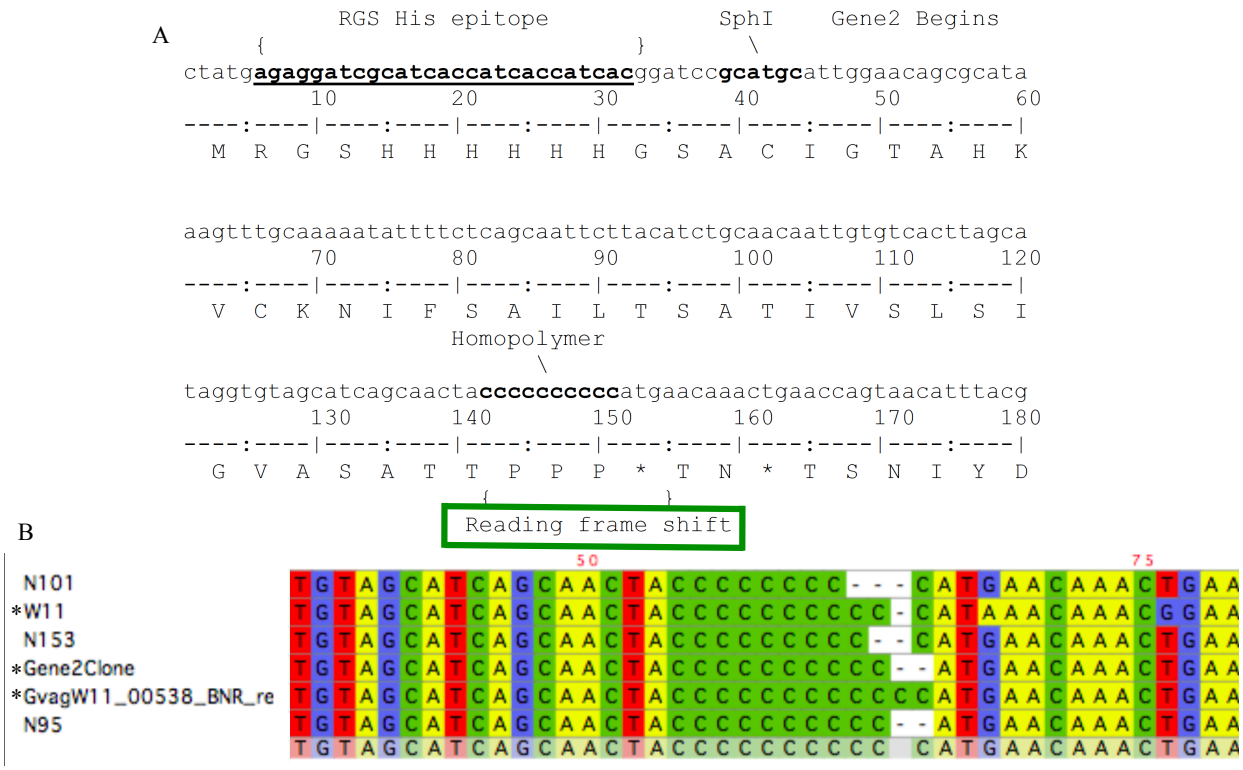

Figure 4.8. Homopolymer sequence analysis.

A) DNA sequence with amino acid translation below showing the beginning of pQE-80L with the six Histidine tag, restriction site *SphI* and the beginning of Gene 2, in which there is a cytosine (n=10) homopolymer region that has caused a reading frame shift. B) DNA alignment of Gene 2 sequences from three other subgroup B isolates, a W11 PCR amplicon, Gene 2 sequence from a clone, and the Gene 2 sequence from the whole genome of W11. (* sequences originate from the same strain).

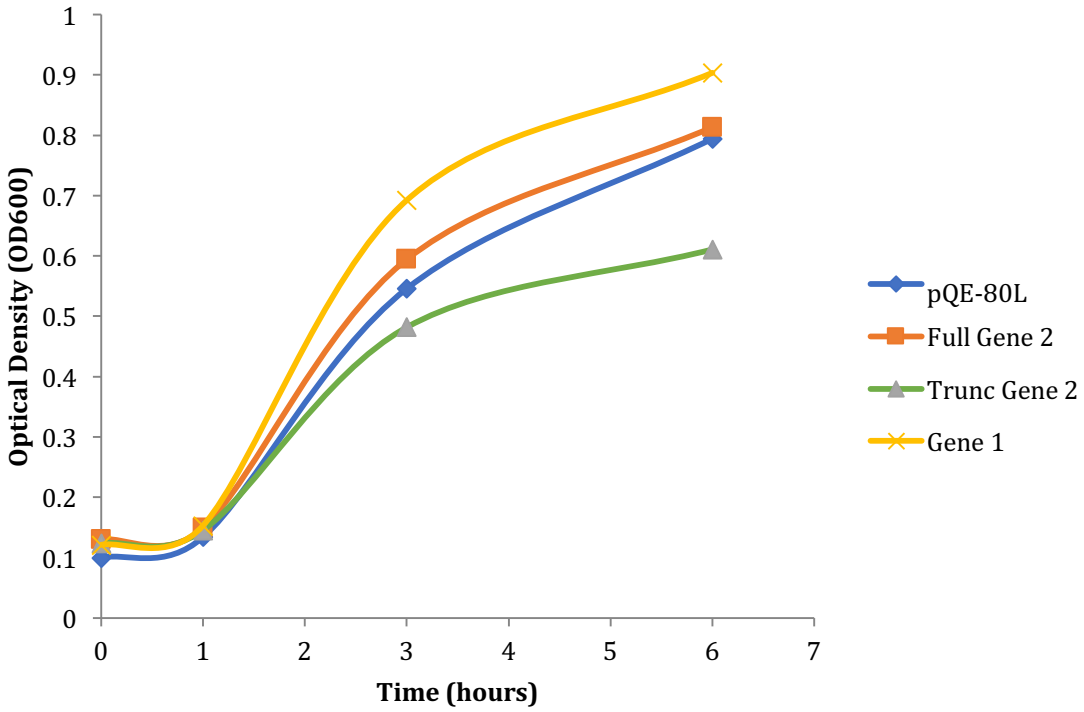

Figure 4.9. Toxicity test of Gene 2 clones.

A growth curve of *E. coli* cell cultures containing empty pQE-80L vector, Gene 1, Full Gene 2 and Truncated Gene 2 this was done to determine if there was cell death or inhibition of growth in Gene 2 clones. The OD_{600} of the cultures was taken at hours 0, 1, 3 and 6 (average OD_{600} of duplicate cultures grown).

4.4.8 Localization of Protein 2

Broth cultures of sialidase activity positive *G. vaginalis* isolates, cell pellets and supernatants from these cultures were tested for sialidase activity using the quantitative assay. Four subgroup B isolates and four subgroup C isolates were included in this test. Subgroup C isolates were included as a negative control. The subgroup C isolates NR042, NR038, VN001 and VN007 did not have sialidase activity in broth cultures, culture supernatants or cell pellets. In subgroup B isolates W11, VN015, VN014 and NR032 sialidase activity was detected in broth cultures and cell pellets, with no activity seen in the supernatant (Figure 4.10).

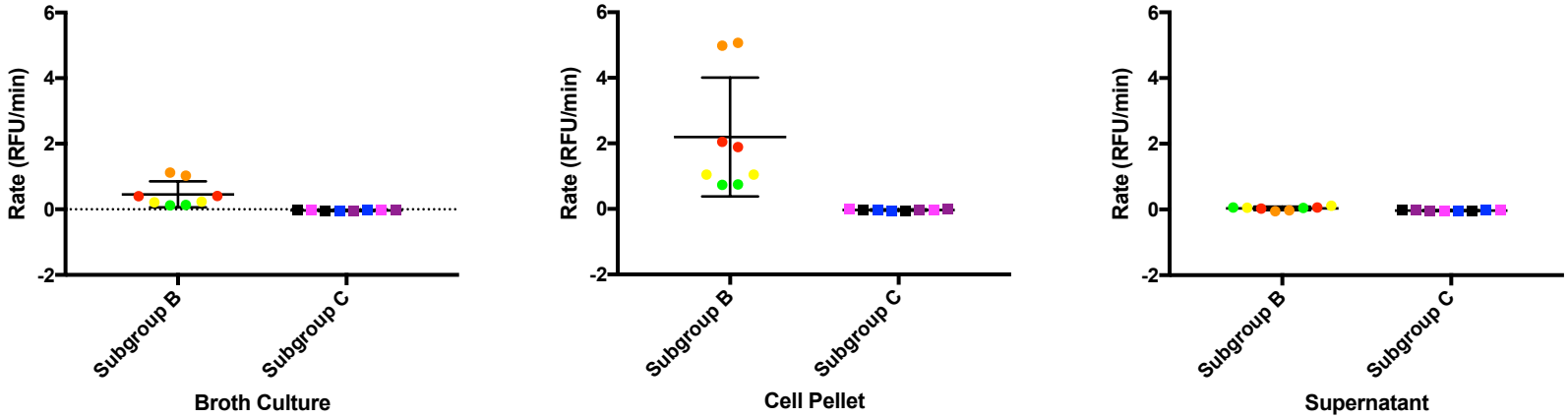

Figure 4.10. Sialidase localization test.

All strains were grown under anaerobic conditions in NYC III broth. Prior to the assay equal volumes of whole cells were aliquoted into two 1.5 ml eppendorf tubes, one was centrifuged to separate whole cells from the culture media. The supernatant was then removed and the cell pellet was re-suspended in an equal amount of fresh NYC III media. Whole cells, re-suspended cell pellet and supernatant were then tested in duplicate for sialidase activity. Duplicate assays are indicated by colour.

4.5 Discussion

The characterization of diversity in *G. vaginalis* subgroups will aid in determining the role they play in the vaginal environment during the development of BV and during symptomatic BV. Many phenotypic and genotypic methods have been utilized to describe the heterogeneity of *G. vaginalis* isolates that have been observed over the years. Speculation about the relative pathogenicity of different strains of *G. vaginalis* has led to investigations of virulence factors including sialidase activity.

The first study identifying that *G. vaginalis* produces a sialidase enzyme was conducted by Von Nicolai et al. (1984). The sialidase was isolated from whole cell cultures that were sonicated to release the sialidase from within the cells, precipitated out of the post-sonicated media and tested for sialidase activity (Von Nicolai, 1984). In that study, there was no identification of a gene that encodes this sialidase, and it is assumed that it is involved with pathogenicity. More recent studies have investigated the association of a putative sialidase gene that was identified with gene annotation in a whole genome (Gene 1) with sialidase activity. Santiago et al. (2011) used ARDRA to genotype over 100 *G. vaginalis* isolates that were also screened for this putative sialidase gene using PCR, and assessed for sialidase activity using a filter-spot test. The authors reported three ARDRA genotypes, two of which had sialidase activity, and they found perfect correspondence of gene presence and activity. This study is limited since there is no knowledge of the *cpn*60 subgroups present and the result may be simply due to a limited sampling of *G. vaginalis* subgroups. In contrast, Pleckaityte et al. (2012) observed that presence of this sialidase gene did not correspond with sialidase activity of 17 *G. vaginalis* isolates examined. Similarly, an examination of a collection of 112 isolates including representatives of all *cpn*60 subgroups

showed that presence of Gene 1 in *G. vaginalis* did not correspond with detection of enzyme activity, and furthermore that sialidase activity was detected only in subgroup B and a few subgroup C isolates (Schellenberg et al., 2016b). One obvious explanation for these observations is that Gene 1 is not responsible for the sialidase activity observed in *G. vaginalis*. Chapter 3 reports the identification of a second putative sialidase gene (Gene 2) that is associated with the presence of sialidase activity. The objective of our study was to express protein from both putative *G. vaginalis* sialidase genes and determine if they encode sialidase enzymes.

Gene 1 was cloned and resulted in successful expression of soluble protein in *E. coli*. Sialidase activity of the purified protein was confirmed using a fluorogenic substrate. This is the first demonstration that this protein has sialidase activity and confirms that Gene 1 does encode a sialidase enzyme (Figure 4.5). Based on the predicted domain structure of the protein product of Gene 1 (Figure 3.2) and its lack of correlation with sialidase activity, Protein 1 is likely an intracellular sialidase enzyme involved in nutrition and utilization of free sialic acids. Intracellular sialidases have been identified in related non-pathogenic bacteria such as *Bifiobacterium longum* subsp. *infantis* (Corfield, 1992), and these enzymes play roles in utilization of free sialic acid as an energy source (Vimr, 2013). Intracellular localization of Protein 1 would explain why some *G. vaginalis* isolates have Gene 1 but do not have extracellular sialidase activity. Furthermore, if Gene 1 is not responsible for extracellular sialidase activity in *G. vaginalis,* detection of Gene 1 by PCR is not an appropriate method on its own for identification of *G. vaginalis* isolates with extracellular sialidase activity.

The pH optimum of Protein 1 was estimated to be 4.5-5, consistent with previously characterized sialidases (Figure 4.6). Bacterial sialidases have been reported to have a pH optimum of pH 5-7 (Corfield, 1992). Tanaka et al. (1992) reported that a sialidase isolated from *Bacteroides*

fragilis had highest activity at pH 6.1, and had activity from pH 6.0 to 6.5. The crude enzyme remained stable and active between pH 5 and 9, purified sialidase did not (Tanaka et al., 1992). The pH optimum of a sialidase from *Heliobacter pylori* was reported to be pH 7.5 (Dwarakanath et al., 1995). The studies conducted in the 1990s do not indicate whether these sialidases are intra- or extracellular. A more recent study reports pH optima of sialidases in *Clostridium perfringens,* which has two extracellular sialidases and one intracellular sialidase (NanH) (Li and McClane, 2014). Li and McClane (2014) reported highest activity of NanH at pH 5.5, followed by less activity at pH 7.2 and significantly lower levels of activity at pH 9.0 (Li and McClane, 2014). This pattern is similar to what was observed for Protein 1, although further experiments will be required to determine if activity continues at pH values above 7.5. These observations of activity at near neutral pH are biologically relevant as cell cytosol is generally around pH 7.0 (Morimoto et al., 2016; Nakamura et al., 2009).

Initial attempts to express the full-length Gene 2 in *E. coli* were not successful under any of the expression conditions tested, due to the presence of a cytosine homopolymer at 141 bp that caused a frame shift resulting in a premature stop codon (Figure 4.8). An alignment of sequences of the homopolymer region revealed variability in its length within the same strain of *G. vaginalis* as well as among other strains. The use of a proofreading polymerase in PCR amplification eliminates PCR error or sequencing artifacts as possible explanations for the variability of the homopolymer region (Figure 4.8). This variability may be due to slipped-strand mispairing (SSM) which is a type of phase variation mechanism used for bacterial gene regulation (Torres-Cruz and van der Woude, 2003). Simple repetitive DNA sequences are common and an abundant feature of genomic DNA, which consist of a variety of repeated motifs 1-10 bases in length (Levinson and Gutman, 1987). The mechanistic basis of SSM was established over 55 years ago by Fresco and

Alberts (1960). SSM requires local denaturation and displacement of the strands of a DNA duplex followed by mispairing of complementary bases at the site of a short tandem repeat (Fresco and Alberts, 1960; Levinson and Gutman, 1987). This SSM mechanism may be important in *G. vaginalis* and more work is needed to investigate this potential regulation mechanism and how it may influence sialidase activity and virulence.

Attempts to express a full-length Gene 2 without the premature stop codon and a truncated version of Gene 2 that did not include the cytosine homopolymer were also unsuccessful with no detectable protein by SDS PAGE of *E. coli* cell lysates. This led to the idea that perhaps the Gene 2 product was toxic resulting in inadequate cell growth or cell death, which would prevent detection of expressed protein. The *E. coli* cultures containing either the truncated or full-length Gene 2 clones grew with no inhibition from toxic or lethal protein products and no major differences were observed (Figure 4.9). Other mechanisms at play that could be influencing expression of Protein 2 include codon usage bias, *E. coli* host strain, and vector selection. These factors will be addressed in future studies in our lab.

Protein 2 is predicted to contain a signal peptide for export and a transmembrane domain at the C-terminus that may provide a cell wall anchor (Figure 3.2). Localization of Protein 2 was investigated using cultures of four *G. vaginalis* subgroup B strains, and four *G. vaginalis* subgroup C strains. Results of these experiments suggest that the extracellular sialidase of *G. vaginalis* is indeed a cell bound protein (Figure 4.10). This is consistent with the results of the first identification of sialidase presence in *G. vaginalis* by Von Nicolai (1984). Von Nicolai detected sialidase activity in both the cell pellet and the supernatant, after releasing the membrane-bound enzyme using sonication. It is important to note that supernatant was concentrated in that study, and we simply may have not had enough protein in the supernatant to reach detectable levels. In

that study, only one out of ten *G. vaginalis* isolates had sialidase activity, which was attributed by the authors to loss of expression from several passages *in vitro* (Von Nicolai, 1984). We now know, however, that some *G. vaginalis* isolates lack sialidase activity due to absence of the gene rather than loss of enzyme production.

Much more recently, Nishiyama et al. (2017), characterized a cell-wall anchored, extracellular sialidase enzyme in *Bifidobacterium bifidum* and demonstrated its role in removing sialic acid from mucin, and promoting adhesion to mucosal surfaces in the gastrointestinal (GI) tract (Nishiyama et al., 2017). Nishiyama et al. (2017) also examined how this exo-α-sialidase benefits the growth of *B. bifidum* in the GI tract making this extracellular sialidase a bifunctional enzyme aiding in cell nutrition and adhesion. A recent report also observed cross feeding between a sialic acid utilizing *B. breve* that lacked an extracellular sialidase and a sialic acid releasing *B. bifidum* (Egan et al., 2014). These results suggest that *B. bifidum* stimulates the production of utilizable sialyloligosaccharides through a "selfish" function and supplies sialic acid or non-sialylated carbohydrates to other *Bifidobacterium* strains through an "altruistic" function (Turroni et al., 2016). These studies demonstrate that sialidases can play roles in cell adhesion, and providing nutrition to other bacteria in the environment. Taken together, the observations here about *G. vaginalis* Gene 2 suggest it has similar characteristics to the cell-wall anchored sialidases of bifidobacteria, raising the possibility that *G. vaginalis* strains with extracellular sialidase activity play important roles in establishment of adhesive biofilm and the proliferation of bacteria associated with BV by providing an energy source from otherwise inaccessible molecules.

4.6 Conclusions

There remains a large amount of work to be done in characterizing *G. vaginalis* and determining its complicated role in the vaginal microbiome and in BV. By confirming that these sialidase genes do indeed encode sialidases we may then better understand the nutrition and possibly pathogenic mechanisms that *G. vaginalis* utilizes them for. Results of this study provide strong support for the hypothesis that Gene 1 encodes a soluble intracellular sialidase protein and that Gene 2 encodes a cell bound sialidase responsible for the observed sialidase activity in *G. vaginalis* subgroup B isolates and a few subgroup C isolates. Future work will focus on achieving expression and characterization of the Gene 2 product through investigation of codon usage, use of other expression vectors and host strains, and expression of functional domains.

5 General discussion

5.1 Summary and limitations of these works

5.1.1 Identification and distribution of Gene 2 in a collection of 112 *Gardnerella vaginalis* isolates correlates with sialidase activity

G. vaginalis has been considered a hallmark organism of BV since it was first identified in the 1950s and through the years of its study has revealed genotypic and phenotypic diversity. Such diversity has been seen in *cpn*60 based phylogenetics of *G. vaginalis* and phenotypically with virulence factors such as sialidase.

Chapter 3 describes the identification of a second sialidase gene (Gene 2) using publicly available whole genome sequences, and demonstrates its association with sialidase activity in a collection of 112 *G. vaginalis* isolates. Gene 2 is almost exclusively found in subgroup B isolates but was seen in three subgroup C isolates in our culture collection where its presence correlated with sialidase activity. These findings reflected the results of our phylogenetic analysis of Gene 1 and 2 sequences from whole genomes, which further suggests that the presence of Gene 2 in a few subgroup C genomes likely occurred due to lateral gene transfer. Based on analysis of amino acid sequences, the protein product from Gene 1 was predicted to be an intracellular sialidase. The Gene 2 protein product was also predicted to be a sialidase, but differed from Gene 1 in that it contained a cytoplasmic domain, a non-cytoplasmic domain, a transmembrane domain and a signal peptide. These predictions are consistent with Gene 2 encoding an extracellular sialidase, and thus being responsible for observed sialidase activity in *G. vaginalis* subgroups B and C. Phylogenetic analysis of peptide sequences of Protein 1 and 2 showed the closest orthologues were found in

Bifidobacterium species. However, there was a Protein 1 homologue identified in 2/127 draft *C. trachomatis* genome sequences and 1/423 draft *N. gonorrhoeae* genome sequences. A Protein 2 homologue was identified in 1/127 draft *C. trachomatis* genomes. The strong similarity of *G. vaginalis* sialidase protein sequences to orthologues in *C. trachomatis* and *N. gonorrhoeae* is consistent with sporadic lateral gene transfer. Since these sialidase genes were observed in so few strains of *C. trachomatis* and *N. gonorrhoeae* this is apparently quite a rare event. The genes were only observed in draft genomes, which did not contain the entire open reading frame sequence of the sialidase. Determination of the complete sequences of these sialidases would strengthen our phylogenetic analysis and further elucidate the relationships of these sialidases.

5.1.2 The protein product of Gene 1 is a sialidase enzyme

The results of experiments presented in Chapter 4 show that Gene 1 does indeed encode a sialidase enzyme in *G. vaginalis*. Activity of Protein 1 was tested using a fluorogenic substrate for qualitative and kinetic assays. The pH profile of Protein 1 showed highest rate of activity at pH 4.5 - 5.0, with relatively low activity at pH 3.5 and 7.5. This pH profile is consistent with previously reported pH optima of sialidases. However, this experiment offers quite a coarse examination of pH optimum as 0.5 increments are quite large and enzymes can be quite sensitive to change in pH.

5.1.3 Protein 2 is likely an extracellular sialidase

Attempts to express Gene 2 in *E. coli* were unsuccessful, however, they led to the discovery of a homopolymer region at the beginning of the gene sequence that resulted in an early stop codon. This homopolymer region was found to be of variable length among other *G. vaginalis* isolates.

DNA repeats such as this homopolymer may be used in slipped-strand mispairing, a form of gene regulation. This homopolymer region was thought to be the reason we did not have successful protein expression. To prevent this homopolymer causing further issues, a primer was designed to amplify a truncated version of Gene 2 that lacked this region. However, no protein was expressed from the truncated version either. A growth curve was performed to confirm that the protein product was not toxic. Several different conditions were tested for expression by altering temperature, incubation time and concentration of IPTG, however this had no impact on Gene 2 clones, which did not express protein under any conditions. Further analysis of the variation of the Gene 2 homopolymer region would aid us in understanding how much it varies and perhaps under what conditions it may change, potentially leading to identification of a novel gene regulation mechanism.

A preliminary localization test was performed with whole cells of *G. vaginalis* isolates (4 subgroup B isolates and 4 subgroup C isolates). This was done by growing *G. vaginalis* isolates in NYC III broth media, centrifuging 1 ml of each isolate, removing the supernatant and re-suspending the cell pellets and testing them for sialidase activity using a kinetic assay. Sialidase activity was strictly associated with the re-suspended cell pellets of the four subgroup B isolates, while subgroup B supernatant, subgroup C cell pellet and culture supernatants were all negative for sialidase activity. These observations, combined with the protein structure prediction described earlier, support the hypothesis that Protein 2 is an extracellular sialidase that is anchored to the cell wall. This observation would be further supported by the demonstration that Gene 2 encodes a sialidase.

5.2 Discussion of future prospects

The work done in Chapter 3 focused on identification of putative sialidase genes in *G. vaginalis* whole genomes and demonstration of sialidase activity correlated with Gene 2 in a collection of 112 *G. vaginalis* isolates. This work is important in characterizing the four *G. vaginalis* subgroups and will aid in identifying the roles each subgroup has in the vaginal microbiome and BV. The pathogenesis of each subgroup is not currently known, and identification of phenotypic traits such as sialidase which is a virulence factor will further our understanding of its ability to colonize, proliferate and persist following antibiotic therapy. Additionally, the observation of *G. vaginalis*-like sialidase proteins in *Chlamydia trachomatis* and *Niesseria gonorrhoeae* and Gene 2 in some subgroup C isolates shows that lateral gene transfer occurs on occasion and may have implications in virulence of other subgroups and vaginal bacteria.

In Chapter 4 the protein encoded by Gene 1 was cloned, expressed and shown to have sialidase activity indicating that this gene encodes a sialidase and has a pH optimum similar to previously reported sialidases. By cloning and expressing protein from Gene 2 we may gain knowledge of the conditions that Protein 2 has optimum activity. Future attempts to express this gene should consider amplification and cloning of catalytic sialidase domains, and different expression vectors. Furthermore, identifying that the Gene 2 *G. vaginalis* sialidase is anchored to the cell wall will aid in providing a better understanding of how this sialidase benefits *G. vaginalis* and the surrounding bacterial population.

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